Spring 2014

Lipidomic analysis of glioblastoma multiforme

Soo Jung Ha
Purdue University, has@purdue.edu

Graduate School ETD Form 9
(Revised 01/14)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Soo Jung Ha

Entitled
Lipidomic Analysis of Glioblastoma Multiforme

For the degree of Master of Science

Is approved by the final examining committee:

Kari L. Clase

Jenna L. Rickus

John A. Springer

To the best of my knowledge and as understood by the student in the *Thesis/Dissertation Agreement. Publication Delay, and Certification/Disclaimer (Graduate School Form 32)*, this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Kari L. Clase

Approved by Major Professor(s): _____

_____

Approved by: James L. Mohler 04/17/2014

Head of the Department Graduate Program Date

LIPIDOMIC ANALYSIS OF GLIOBLASTOMA MULTIFORME

A Thesis

Submitted to the Faculty

of

Purdue University

by

Soo Jung Ha

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

May 2014

Purdue University

West Lafayette, Indiana

To my parents – for loving me and supporting me unconditionally and trusting all my decisions.

To my sister, Jinjoo – for being my best friend and keeping the conversations with me during countless sleepless nights.

To Jim Piersma – for supporting my career and being there for me every step of my graduate career.

And to my grandmother – for loving me and protecting me from the other side.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Kari Clase, for her guidance and support throughout my master's career. Dr. Clase exposed me to many valuable experiences and helped me to find my passion in research. I would also like to thank my committee members, Dr. Jenna Rickus and Dr. John Springer, for their input and advice. They never hesitate to guide me with the projects and provided ideas with their expertise.

To my family, I could not thank enough for their love and support. I also thank them for trusting all my decisions. To Jim Piersma, I thank him for encouraging me with my career, dealing with my frustrations, and being there for me every step of the way in my journey.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Ha, Soo Jung. MS, Purdue University, May, 2014. Lipidomic Analysis of Glioblastoma Multiforme. Major Professor: Kari L. Clase.

Glioblastoma Multiforme (GBM) is the most common and malignant form of the primary brain tumor. Due to its highly invasive nature, current treatment options have not been able to improve the survival rate in past 20 years. In order to discover GBM therapeutic targets, omics technologies have been widely used to identify potential biomarkers.

This research study focused on investigating lipid biomarkers of human GBM orthotopic mouse models employing mass spectrometry. Human tumor cell lines GBM10 and GBM43 were injected in the right cerebral hemisphere and flank sites in NOD/SCID mice (n = 10 mice per group). Left cerebral hemispheres of the mouse brains were harvested as control tissue. After harvesting brain and flank tumors and control brain from the xenograft models, protein, metabolites, and lipids of tumor samples were collected through the simple extraction procedure. These samples were analyzed by reverse phase high performance liquid chromatography – Fourier transform ion cyclotron resonance mass spectrometry (RHPLC-FTMS). FTMS analyzers have the highest resolving power

of all MS instruments, which is ideal for complex mixtures such as GBM tissue. Spectra obtained from the FTMS analysis were analyzed using Student t-tests to detect significant differences in tissue profiles at a level of $p = 0.05$. Compounds below this threshold were identified through a database using the m/z ratio.

Lipidomic analysis indicated the possible differentially expressed lipids classes in GBM tissues, and connected to metabolic pathways, tumor proliferation and immunodepression. Most significantly expressed lipids were glycerophospholipids, glycerophosphocholines, glycerophoserines, and triradylglycerols. Accompanying these studies is a collaborative effort to improve the effectiveness and efficiency of computational pipelines that are imperative to the analytics, visualization, identification, and interpretation of the omics data. Only by carefully integrating the computational pipelines can we successfully perform the types of integrative studies needed to advance the identification of cancer biomarkers for diagnosis and prognosis, and our integrative studies serve as a case study for our pipeline advancement efforts.

CHAPTER 1. INTRODUCTION

This chapter introduces the overview of lipidomic analysis of Glioblastoma Multiforme (GBM) research study. This chapter provides a statement of purpose in GBM cancer studies, research questions, scope and significance of the study that explain needs of knowledge in cancer lipids, definitions of key terms, assumptions, limitations, and delimitations of this research study.

1.1 Statement of Purpose

Cancer has become one of the most common diseases in people's lives. Although many scientists attempt to find the effective way of treatment for cancer, the success rate has not dramatically increased over the past several decades. Glioblastoma Multiforme (GBM) is one of the most aggressive types of cancer that occurs in the human brain. Current treatment options of glioblastoma have not increased survival rates of GBM patients beyond 20 years. The purpose of this research is to discover potential cancer targets of GBM by using cutting edge technologies to investigate proteins, metabolites, and lipids of orthotopic and subcutaneous xenograft mouse tumor tissues. Figure 1.1 overviews the general cancer proteomics workflow and therapeutic goals using different existing models. However, studies of proteins have not been sufficient to identify effective

targeting biomarkers. This experiment includes analysis of metabolites and lipids of GBM to explain distinct characteristics of GBM along with protein biomarkers. This thesis covers the lipidomics part of the project.

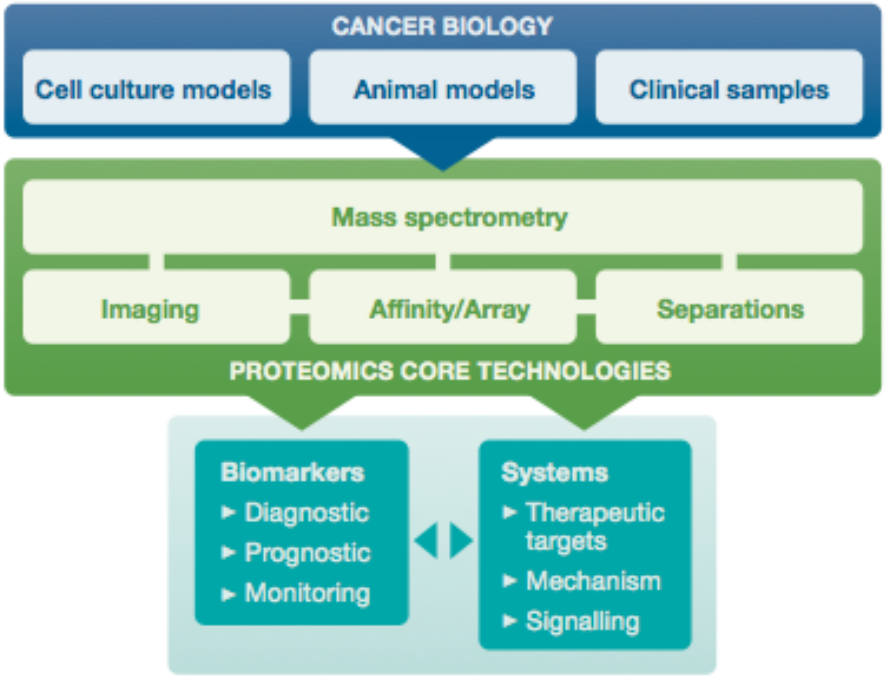


Figure 1.1. Generalized workflow for cancer proteomics studies using model systems and clinical samples *(Collins et al., 2009).*

Another important goal of this study is the comparison of various existing cancer models to explain the morphology changes of cancer cells in different microenvironment and reliability of the models. Cancer models such as human in vitro, mouse in vivo, and naturally occurring animal cancer play critical roles in cancer research. This research study utilized orthotopic and subcutaneous xenograft mouse models and two different human GBM cell types (GBM10 and GBM43) to compare and identify the lipid composition of GBM. Furthermore,

these results were examined to discover metabolic characteristics of the GBM that can potentially be compared with metabolite and protein data in the future.

1.2 Research Questions

- What are the similarities and differences of molecular profiles between brain (orthotopic) and flank (subcutaneous) tumors in mouse xenograft model?
- Are there biomarkers in GBM10 and GBM43 that are related to metabolic properties of GBM?

1.3 Scope

The scope of this study was lipidomic analysis of Glioblastoma Multiforme (GBM) using electrospray ionization Fourier transform mass spectrometry (ESI-FTMS; 7 T Bruker Instrument) and various data analysis tools. The research focused on the characterization of lipids in orthotopic and subcutaneous mouse xenograft models using comparative analysis to investigate the characteristics of GBM that can possibly provide evidence in the unique patterns of GBM metabolism. Furthermore, this data explained the interactions between metabolite and protein profiling data for future work. Global lipid profiling data from mass spectrometry was analyzed using various bioinformatics tools (MzMine2 and LIPID MAPS) and statistical method to identify the significant lipid contents from the xenograft tumor samples.

1.4 Significance

Technology plays a significant role in disease studies, including cancer research. Omics studies have been employed in disease studies to investigate the characteristics of the disease and to discover novel biomarkers. According to The Cancer Genome Atlas, more than 500 human GBM tumors have been sequenced and characterized (Brennan et al., 2013). In addition to active investigation of GBM genomics, whole proteome analyses were performed using various human samples in the past decade (Niclou, Fack, & Rajcevic, 2010). Even though genomics and proteomics provide knowledge of GBM, these studies fail to suggest effective targets for therapy.

Lipidomics is a relatively new field, introduced in 2003 by Han and Gross (Niclou, Fack & Rajcevic, 2010). Lipids in GBM have not been studied as much as proteins and genomes due to biological and technical challenges. However, lipids have many important functions in biological systems, such as energy storage, structural components, cell membrane composition, and cell signaling. Moreover, human brain tissue consists of five to 15% lipids, the highest amount of lipids in comparison to other organs within the body (Campanella, 1992). Lipid metabolism regulated by oncogenic signaling pathways is known to play an important role in cancer initiation and progression (Zhang, 2010). However, the role of lipids in cancer studies is still poorly understood due to biological and technical difficulties (Shevchenko & Simons, 2010; Tripathy, 2011). As a result of lack of lipidome information, genomic and proteomic profiles have not been integrated with lipidomics. The majority of lipid metabolism in cancer studies

investigate protein levels of tumor tissues in order to predict the lipid function and composition. These studies also tend to focus on the importance of lipid synthesis.

In order to explore discovery lipidomics, the two most common types of xenograft mouse models were used to generate GBM tumors from human GBM cells lines. Human GBM10 and GBM43 that were provided by Mayo Clinic, Rochester, Minnesota, were injected into both subcutaneous (flank) and orthotopic (intracranial) sites. Tumors from different xenograft models may represent different histopathologic, genetic, and growth properties of GBM. From our data, we hypothesized that fatty acid oxidation plays a critical role in glioblastoma development and malignancy of the tumor as much as lipid synthesis. To test this assumption, proteomic and metabolomic data will be used to validate the metabolic patterns in GBM. This study is also a collaborative effort to improve the effectiveness and efficiency of computational pipelines that are imperative to the analytics, visualization, identification, and interpretation of the omics data through integration of existing analytical algorithms. Integrative studies serve as a case study for our pipeline advancement efforts and identification of therapeutic biomarkers.

Integrated omics study can characterize the distinct biological properties of GBM that can lead to effective way of targeting malignant brain tumors. This thesis covers the lipidomic element of the project. A novel extraction method used to collect proteins, metabolites, and lipids in a single sample preparation resolves the challenges in biological sample preparation. ESI-FTMS allows

different biological levels of samples to generate the global profiling data with the highest sensitivity and mass accuracy among existing instruments. Another important goal of this study is to compare existing models to investigate the similarities and differences between different GBM models to improve the cancer modeling system and justify how well these models can mimic human GBM. The results of the study provide insights into the improvement of outcomes in the discovery of novel GBM biomarkers and a GBM modeling system.

1.5 Definitions of Key Terms

apoptosis – a distinct process of cell death that is responsible for cell death (Kerr & Winterford, 1994).

biomarker – molecular indicator that distinguish normal or diseased process states in the body (Tainsky, 2009).

carcinogen – compound that causes formation of DNA adducts. It has an ability to initiate various genetic mutations that cause cancer (Herbst et al., 2008).

lipidomics – system-level identification and quantification of pathways and networks of cellular lipids, molecular species, and interactions (Tripathy, 2011).

mass spectrometry – the study of matter that is transformed into gas-phase ions and detect by mass/charge ratio (Murray et al., 2013)

metabolites – small molecules that have functions in cellular state and are chemically transformed in metabolism (Patti, Yanes, & Siuzdak, 2012).

metabolomics – the global quantitative study of metabolites using omics technologies that utilizes the analytical instrumentation with pattern recognition techniques to investigate changes in metabolites (Beger, 2013).

metastasis – cancer cell detachment from the primary tumor site and migration to other body sites through the lymphatic or blood circulatory systems (Chiang & Massague, 2008; Gupta & Massague, 2006).

oncogene – a gene that is mutated by carcinogens and causes uncontrollable cell growth that becomes cancer cells (Herbst et al., 2008).

proteomics – technology-driven scientific study of proteins, especially their changes, proteomes, post-translational modifications, and interactions (Fountoulakis, 2001).

Temozolomide (TMZ) – a DNA alkylating agent that demonstrates antitumor activity used for glioma treatment (Stupp et al., 2005).

tumorigenesis – genetic alterations that generate the progressive transformation of normal cells into a highly malignant tumor (Hanahan & Weinberg, 2000).

xenograft – in cancer studies, human tumor cell transplantation into immunocompromised mice that do not reject human cells (Richmond & Su, 2008).

1.6 Assumptions

The following assumptions are inherent to the pursuit of this study:

- In vivo tumor cells are not contaminated by microbial contaminants.

- Lipid profile data that is generated by mass spectrometry is accurate.
- In vivo tumor environment can change morphology of GBM.
- Lipid database matches theoretical mass/charge ratio of lipids from GBM.

1.7 Limitations

The following limitations are inherent to the pursuit of this study:

- Human GBM10 and GBM43 were used to generate tumors in mouse xenograft models and generate data.
- Orthotopic and subcutaneous mouse xenograft tumors were used as different cancer models.
- MzMine2 and LIPID MAPS were used to identify the significant lipids of GBM.

1.8 Delimitations

The following delimitations are inherent to the pursuit of this study:

- Not all types of GBM samples were used to represent GBM results.
- There was no comparison of results using different bioinformatics tools other than MzMine2 and LIPID MAPS.
- Existing cancer models other than mouse were not examined for the comparison.

1.9 Summary

This chapter has provided an overview of the research study, including statement of purpose, research question, scope, significance, definitions of key terms, assumptions, limitations, and delimitations. The next chapter provides an overview of glioblastoma, current treatment options, proteomics, metabolomics and lipidomics in cancer, biomarker discovery, and animal models of GBM.

CHAPTER 2. REVIEW OF RELEVANT LITERATURE

Glioblastoma (GBM) is known as an aggressive form of brain tumor that has a low survival rate. This chapter provides an overview of glioblastoma, current treatment options, and research focus. It introduces current and future treatment options of GBM and optimization of animal models to understand the characteristics of human GBM. Characteristics of omics studies are also discussed.

2.1 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is the most common form of primary brain tumor (Pelloski & Gilbert, 2007). GBM is also called a grade IV astrocytoma (Kleihues, Burder, & Scheithauer, 1993). GBM represents about 50% of all gliomas and is a distinct primary tumor type (Jellinger, 1978). Figure 2.1 shows the distributions of primary brain and CNS tumors (figure 2.1 A) and primary brain gliomas in the United states from 2004 to 2006 (figure 2.1 B). Despite many treatment options, it is still incurable and has less than a two-year survival period from the time of diagnosis. GBM can occur at all ages and most frequently in the elderly (Ohgaki et al., 2004). Progression of gliomas shows genetic and epigenetic alterations, including the loss of tumor suppressor gene function

(PTEN, TP53, INK4A/p16, ARF/p19, Rb) or activation of oncogentic pathways (p21-Ras, PI3-kinase, EGFR, CDK4, MDM2) (Agnihotri et al., 2013).

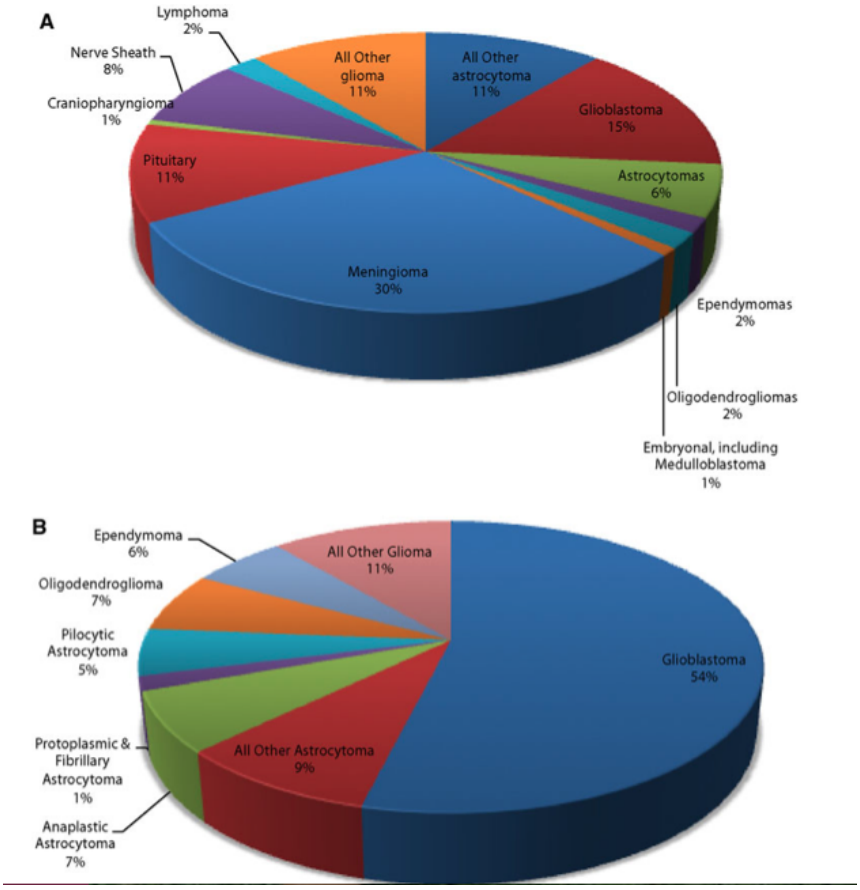


Figure 2.1. Primary brain tumor distributions (Agnihotri et al, 2013).

Glioblastoma Multiforme is highly invasive and malignant and the tumor cells migrate great distances from the primary tumor site by its diffuse infiltration characteristic. Tumor cells disseminate from the primary tumor site and migrate along the white matter tracks, the basal lamina of brain blood vessels, or in between the glia limitans and the pia matter. Invasion of GBM steps include sequential adhesion to the extra cellular matrix (ECM) using CD44 and

hyaluronan-mediated motility receptor (HMMR), degradation of the ECM, and altered cell contractility and motility (Agnihotri et al., 2013).

2.2 Treatment Options in Glioblastoma

Prognosis of Glioblastoma (GBM) have not been changed over several decades even though diagnostic modalities, surgical techniques, and adjuvant treatment strategies have been developed dramatically in the last 30 years (Oertel, von Buttlar, Schroeder, & Gaab, 2005) . GBM has a median survival time of approximately 12 to 15 months from diagnosis. The two-year survival rate is only about five to 15% (Stupp et al., 2005). Due to the extremely aggressive nature of GBM, current treatment options are not designed to improve patients' quality of life, but simply to extend their life (Lipsitz et al., 2003). There are several treatment options for glioma treatment.

2.2.1 Current Therapies

Surgery may eliminate some tumor tissue using maximal resection and intra-operative resection (local treatment) (Pelloski & Gilbert, 2007). The study shows that surgery may extend survival times approximately 14 weeks. However, GBM surgery is very difficult because of GBM's invasive, diffuse, and poorly defined borders of the tumor (Agnihotri et al., 2013). Autopsy studies show that malignant cells are found in brain distant from the primary site (Pelloski & Gilbert, 2007). GMB is incurable by surgery in the majority of patients due to more than 80% of recurrence rate within the original tumor site. However, the surgical

method is important for managing the tumor in patients. Radiotherapy and chemotherapy are usually administered after surgery to increase survival rate of GBM patients (Reardon & Wen, 2006).

Due to difficulties of design and implementation, only a few prospective surgical trials have been attempted. A surgical design utilizing 5-aminolevulinic acid has been recently attempted in Germany. This method uses 5-aminolevulinic acid in order to help surgeons perform maximized resection of contrast-enhancing portion of GBM (Pichlmeier et al., 2008). However, current surgical methods have not increased patients' overall survival period dramatically.

Radiation is broadly used to decrease the GBM tumor site in the brain and slow the process of cancer and it is commonly coupled with the surgery method. However, GBM is difficult to treat with radiation due to GBM's tendency to widely infiltrate brain tissues. (Pelloski & Gilbert, 2007). No perspective radiation studies have been attempted and existing techniques, intensity-modulated RT (IMRT) and traditional 3-dimensional EBRT, have not shown improved results in GBM therapy (Clarke et al., 2010).

Another brain tumor therapy that is used most commonly is chemotherapy. Temozolomide (TMZ) is the most widely used GBM chemotherapy drug that has great oral bioavailability, no significant drug-drug interactions, and no cumulative myelotoxity. This chemotherapeutic agent has a potential to improve overall survival in human GBM patients. TMZ increased the long-term survival rate from 10.4 to 26.5%.

Investigation of TMZ combination with other cytotoxic chemotherapies and with cytostatic agents may increase survival period. However, resistance of GBM to chemotherapy is common (Reardon & Wen, 2006).

2.2.2 Advances in Therapies

Despite these treatment options, recurrent glioblastoma patients gain only minimal or modest improvement from current treatments. The objectives of GBM therapy investigation are to target cellular pathways or specific biomarkers in pathogenesis and to identify molecular properties of cancer that predict a therapeutic response. On-going GBM research focuses on inhibition of tumor function, survival, proliferation, apoptosis, invasion, and angionesis (Agnihotri et al., 2013). Much current research investigates the neural signaling pathway of glioblastoma to block tumor growth. Knowledge of signaling pathways has elucidated new potential therapeutic targets. A large number of new glioblastoma drugs target these signaling pathways. Since proliferation and survival pathways are mostly regulated by growth factors and their receptors, inhibition of these pathways and receptors can eliminate and prevent tumor formation in brain. There are also many studies to inhibit angiogenesis of tumors, Ras/MAPK and PI3K/Akt pathways, proteasomes, and histone deacetylases (Reardon & Wen, 2006).

Another advance in the treatment of glioblastoma is an antiangiogenic therapy. The formation of new blood vessels, angiogenesis, plays an important role in tumor growth. Tumor growth and spread are sustained by oxygen,

nutrients, and growth factors through blood vessels (Hamahan & Folkman, 1997). In malignant gliomas, angiogenesis is associated with an increase in vascular endothelial growth factor (VEGF). VEGF is a protein that promotes formation of new blood vessels, which stimulates endothelial cell growth, migration, and survival from preexisting blood vessels (Hicklin & Ellis, 2005). There are several drugs that are on trial for malignant gliomas. Bevacizumab is a monoclonal anti-VEGF antibody that is in several phase 2 trials. However, Bevacizumab has serious toxicity that could promote a more invasive aggressive tumor and shorten survival period (Stupp et al., 2009). Cilengitide is an inhibitor of αvβ3 and αvβ5 integrin receptors, which are activated by VEGF and promote angiogenesis (Meredith et al., 1993). It has been investigated in phase 2 trials with and without radiation/Temozolomide on recurrent GBM (Stupp et al., 2007). Another drug that is in trial for antiangiogenesis is Talampanel. Talampanel is a glutamate receptor blocker that inhibits the proliferation and migration of GBM. It showed statistically significant improvement in GBM patients and will be investigated in a phase 3 trial (Grossman et al., 2009).

A variety of other cancer therapeutic approaches are under active investigation. Gene therapy uses the insertion or modification of genes into a cell to treat cancer. Gene delivery can be performed using vectors from viruses or synthetic vectors such as nanoparticles. However, clinical trials have been limited due to a short distance of the delivery site (Clarke, et al., 2010).

Immunotherapeutic therapy has been studied for glioma. There are two types of immunotherapy: active immunotherapy and passive immunotherapy.

The goal of active immunotherapy is to promote long-term immune responses by up-regulating immunity against the tumor. Long-term immunity could potentially prevent future tumor recurrence (Clarke, et al., 2010). Tumor vaccines such as EGFRvIII and dendritic cells are in trial to improve GBM treatment (Luptrawan et al., 2008; Choi et al., 2009). Passive immunotherapy attempts to achieve an immediate effect of immune response for short-term immunity by transferring immune effectors. Antibody-mediated drug delivery has been used to increase the local drug concentration and minimize nonspecific systemic exposure (Mitchell & Sampson, 2009). Although there are various approaches of GBM treatment, many treatment options are still in trials and they have not led to a significant increase in the survival period of GBM patients.

2.3 Animal Models of Glioblastoma

Proteomic studies using clinically relevant model systems have potential to discover novel cancer target molecules. Cancer models are investigated to understand disease progression, tumor adaption, and treatment responses. Animal models help researchers to design more controlled experiments, including reproducible sampling, duplicable experiments, and tumor progression over different time periods. Although models have several advantages in clinical research, few glioma studies analyzed animal models (Niclou, Fack, & Rajcevic, 2010).

2.3.1 Current Animal Models

Human/rat xenograft glioblastoma models are commonly used to identify the protein expression in highly malignant, non-angiogenic brain tumors (Niclou, Fack, & Rajcevic, 2010). Figure 2.2 shows the types of mouse models in cancer research, advantages, and disadvantages among different mouse models. The features of ideal GBM models should include good representation of human GBM, gene, and metabolism alteration, reproducibility, and similar tumor progression progress (Yi, Hua, & Lin, 2011). These models are also required to mimic physiological tumor traits, including tumor invasive growth, neovascularization, necrosis, and psedopalisading cells (Miura et al., 2010).

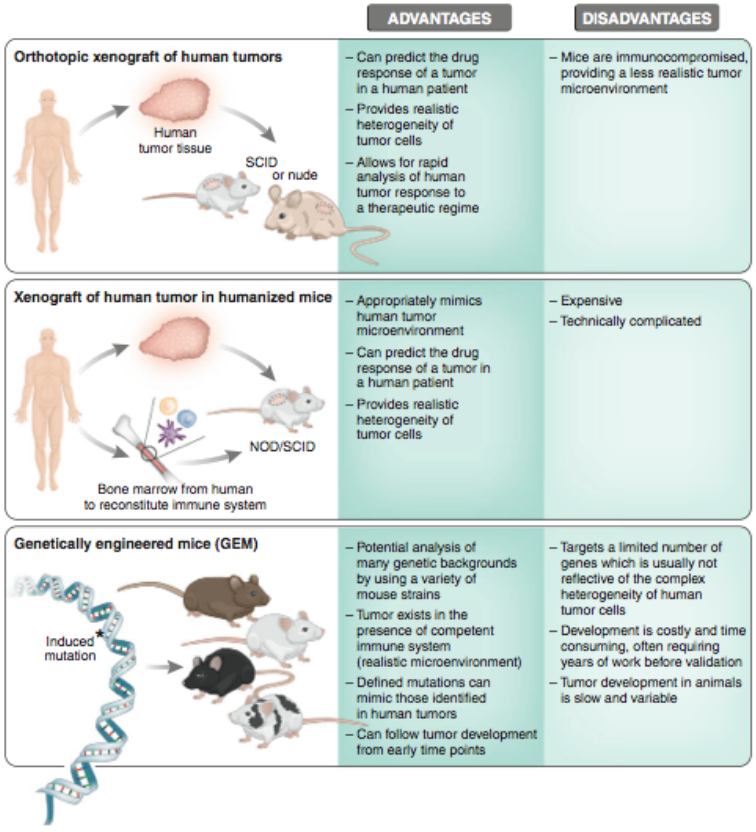


Figure 2.2. Types of cancer mouse models (Richmond & Su, 2008).

Current GBM mouse models are highly efficient for tumor formation, appropriate tumor growth rate, and an accurate tumor growth site. Although mouse models have been playing important role in GBM research for over 30 years, these models have had limitations in providing important biological and pathological properties of human GBM (Yi, Hua, & Lin, 2011). The xenograft tumor models lose genomic and phenotypic properties of human tumor (Martens et al., 2008; Sausville and Burger, 2006; Taillandier et al., 2003). In the result, these models does not represent GBM heterogeneity (Bonavia et al., 2011; Lee et al., 2006; Verhaak et al., 2010).

Rats and rodents are good models to understand human cancer as they are cost effective and time efficient (Pang & Argyle, 2009). However, rat models have not been effective to improve human GBM treatment due to the physiological differences between humans and rats. Successful treatment from rodents often does not translate into the same success in human patients (Pang & Argyle, 2009). Mice models show similar results as rat models. Many drugs that are effective treatments for mice show low success rates in human clinical trials due to low efficiency or toxicity to human patients.

In this research two different xenograft tumors, flank tumors and brain tumors, were used to discover potential biomarkers, identify distinct patterns of metabolites and lipids of GBM, and to explore the similarities and differences of mouse tumors and human GBM. Flank tumors usually have excessive tumor growth and low immune response on the tumor site (Speroni et al., 2009). Flank tumor also does not represent invasive growth of tumor (Yi, Hua, & Lin, 2011).

Orthotopic tumors have higher cellular influx and higher local immune response. Compared to flank tumors, orthotopic tumors are smaller and have shorter survival rates. These tumors are also highly vascularized (Speroni et al., 2009). Although orthotopic models provide metastatic properties, they often fail to represent the traits of human GBM invasion, diffuse infiltration of the cells, and gene alternations (Speroni et al., 2009; Yi, Hua, & Lin, 2011).

2.3.2 Naturally Occurring Cancer Animal Models

Using spontaneous mammary cancers of other species would be suitable as a better model than rodents for human cancer and enhance understanding of human carcinogenesis (Cocola et al., 2009). Naturally occurring cancers in dogs or cats can be suitable models for study of human glioblastoma on several different levels. Cancer in dogs and humans shares strong anatomical and physiological similarities, including histological appearance, tumor genetics, molecular targets, biological behavior, and response to therapies. Cancer-associated genetic mutations that promote cancer progression in humans have been found in canines. Canine and human cancers also share initiation and progression factors, including age, nutrition, gender, reproductive status, and environmental exposures. Due to the physiological similarities between dogs and humans, several human chemotherapy treatments have been used in veterinary medicine as cancer treatment. According to interrogation of the genome sequence, approximately 19,000 canine genes match to similar or orthologous genes in humans. Several important human cancer-related genetic mutations

and molecular signaling pathways have been identified in canine cancers (Paoloni & Khanna, 2007).

Canine models are able to generate mammospheres and tumourspheres. These models have the capacity to be long-term non-adherent cultures and generate more complex structures. Canine models also allow for the propagation and enrichment of cancer stem-like cells (Pang & Argyle, 2009). The goal of studying spontaneous cancer models is to compare for gene identification, discover environmental risk factors, understand tumor formation and progression, and develop new therapies (Argyle & Khanna, 2006). Accommodation of canine GBM models for new drug studies may improve human cancer trials to discover new cancer drugs.

Naturally occurring canine cancer models are necessary to demonstrate the targeting specificity of cancer treatment within a naturally heterogeneous tumor environment. Naturally occurring canine models can provide the ability to evaluate the potential toxicities and efficacy of this drug and find novel cancer treatment (Paoloni & Khanna, 2007).

2.4 Proteomics

Proteomics is technology-driven scientific study of proteins, especially their changes, proteomes, post-translational modifications, and interactions (Fountoulakis, 2001). Proteomics focuses on investigating the structure and function of the proteins of the human genome, including the delineation of signaling networks and the regulation of the cell function (Khalil, 2006). There are

approximately 35,000 genes in the human genome and theoretically 500,000 to 1,000,000 proteins can be translated (Galvao et al., 2011).

Proteomics plays an important role in characterizing cancer addition to genomics. Since genomics does not directly translate to proteins, mRNA expression data alone is insufficient to predict cellular functions. Addition of proteomic data provides access to a global view of molecular changes in different stage of diseases. Many disease studies attempt to understand changes in protein levels of functions to improve and discover new treatment options. Existing drug mechanisms focus on inhibiting the disease-related protein activities (Khalil, 2006).

Proteomics research focuses on two strategies. The first strategy of proteomics is to define protein-protein interactions in order to investigate complex networks of intracellular signaling pathways. The second strategy is to monitor large-scale global expression of different proteins and quantitatively identify the changes of expression patterns (Simpson & Dorow, 2001). Investigations of global protein expression are conducted in disease studies to identify biomarkers for diagnosis, prognosis, and for identification of therapeutic targets, especially in cancer research.

2.4.1 Cancer Proteomics

Proteomic approaches are widely used in cancer studies. The goal of proteomic analysis in cancer research is to identify new biomarkers for diagnosis and classification of cancer and for defining targets for more-effective therapeutic

outcomes (Simpson & Dorow, 2001). Moreover, discovering pathways of cellular signaling networks has a potential to eliminate tumor initiation and progression (Collins, 2009).

Cancer proteomics mainly investigates increased proto-oncogene expression, inactivation of tumor suppressor genes, chromosomal instability, alterations in DNA repair genes, telomerase reactivation, structural proteins, signal-transducers, cell-cycle regulators, and epigenetic alterations that result in disregulation of cell proliferation, clonal selection, and tumor formation (Galvao et al., 2011; Rodenhiser & Mann, 2006). Quantitative value of protein expression in a proteome provides the information on the cell response due to changes in its cellular environment. The results of these changes are up- regulated or down-regulated proteins, which may regulate cellular activities related to tumor initiation, progression, and metastasis (Khalil, 2006). Information about oncogenic and tumor suppressor proteins and growth factor receptor signaling pathways has been provided through proteomics. Proteomics-driven cancer research has the potential to define protein-protein interaction networks that lead to tumor deregulation and inhibition of cancer progression (Collins, 2009).

2.4.2 Cancer Biomarkers

Biomarkers are molecular indicator of a biological status. Biomarkers can be detected in the blood, body fluids, or tissues. Discovery of biomarkers in cancer is important for several reasons: early detection of cancer, diagnosis, prognosis, response to therapy options, and cancer recurrence. It also improves

decision making for appropriate patient treatment. Tumor biomarkers express cancer-specific mutations, or changes in gene expression or promoter methylation. These changes can result in alterations in protein expression. Cancer-specific protein alterations can be expressed in the protein abundance or the modification of post-translational proteins (Tainsky, 2009). The concentration of specific biomarkers is highest in the tumor cells and its microenvironment (Hondermarck, 2003; Simpson et al., 2008) and combination of several different markers affect tumor behavior (Alaiya et al., 2000).

There are three main useful markers in cancer: diagnostic, prognostic, and predictive markers. These markers provide tumor information about the malignant potential and the prognosis. Diagnostic markers are used to help histopathological classification. Prognostic markers include hormone receptors, proliferation markers, proteases, and angiogenesis markers. These markers are used in diagnosis of cancer. Predictive markers are used to decide different therapy options (Alaiya et al., 2000).

Proteome analysis of glioma has been attempted in different models such as human patients' biopsies and body fluids, human glioma in vitro cell lines, and animal models to discover new treatment targets. Proteins that were discovered to be significantly up-regulated due to brain tumor grade include GFAP, IGF-binding protein 2 (IGFBP2), IGFBPS, PBEF1/NAmPRTase (Nicotinamide phosphoribosyltransferase, plasminogen activator inhibitor-1 (PAI -1), Cathepsin-D, YKL-40, MMP9 and low MW Caldesmon (1-CaD). According to the literature, the GBM pathways that are disrupted are the Ras/MAPK, the PI3K/Akt, the

retinoblastoma and the p53 pathways (Niclou, Fack, & Rajcevic, 2010). Identification and characterization of protein expression changes in the cells have potential to discovery the target biomarkers of cancer (Tainsky, 2009).

2.4.3 Limitations

Proteomics already plays a huge role in studies of mechanisms of tumor formation and identification of proteins for cancer diagnosis and treatment targets (Collins, 2009). However, there are still challenges due to several reasons. There are two major limitations on the cancer biomarker discovery. First, diverse populations of human samples that are collected under various clinical conditions are required. Second, discovery phase studies use the predominant retrospective samples (Zhang & Chan, 2005).

The followings are the direct implications of these two biological facts:

- Complexity of clinical specimen proteome: Human proteomes are complex and dynamic. It is important to develop techniques that are less complex and with narrower dynamic ranges.
- Biological variability: Samples from different populations are significant. The disease-associated proteomic expression data could be significantly different from other expression data.
- Preanalytical variability: There is insufficient protein information for diseases. Proteins samples are often collected under different protocols. The sample handling and processing may be different for the disease and samples.

- Analytical variability: Minimization of analytical procedures variability is necessary (Zhang & Chan, 2005).

There are also several difficulties due to technical challenges:

- The lack of sensitivity and specificity of detecting low abundant biomarkers and complex biological samples (Rai & Chan, 2006).
- Limited detection technology of genome fraction (Alaiya et al., 2000).

2.5 Metabolomics

Metabolomics is the global quantitative study of metabolites using omics technologies that utilizes the analytical instrumentation with pattern recognition techniques to investigate changes in metabolites. Metabolomic profiling is capable of detecting and quantifying metabolites in tissues and biofluids that are associated with biological pathways (Beger, 2013). Metabolomics also has been employed in discovery of functions of genes and proteins. Findings of metabolomics provide insights into biological processes (Patti, Yanes, Siuzdak, 2012). Metabolites play important roles in cellular functions, including cell signaling, energy transfer, and cell-to-cell interaction (Niedbala et al., 2009). Metabolite profiles can be important markers of physiological and pathological states. Analysis of metabolites has the potential to solve the questions on the mechanism of disease occurrence and progression (Zhang & Du, 2012).

Metabolomics measures and monitors changes of small molecules that are up- or down regulated by cell processes (Niedbala et al., 2009).

Metabolomics in cancer research plays a critical role in understanding glycolysis of tumor cells, which is called the “Warburg Effect”, production of the amino acids, nucleotides and lipids that are required for tumor proliferation and vascularization by recognizing the patterns of metabolites. The clinical goal of metabolomics is to discover cancer biomarkers that are used for diagnosis and prognosis. Applying various analytical techniques, metabolomics is able to correlate metabolites and biological pathways in order to identify more accurate potential biomarkers (Beger, 2013). This research study applied mass spectrometry in cancer metabolomics. Using mass spectrometry in metabolomics, thousands of metabolites can be rapidly measured with only minimal amount of samples (Patti, Yanes, & Siuzdak, 2012).

Metabolomic analysis using mass spectrometry has four main steps. Firstly, tumor samples are collected and extraction is performed on the biological sample to harvest metabolites. Secondly, data is acquired by mass spectrometry. Thirdly, bioinformatics tools are used to analyze the data (Beger, 2013). Current metabolomics software, such as XCMS, does not output metabolite identifications. These bioinformatics tools provide p-values and fold changes in intensity between samples. After organizing the metabolites data using bioinformatics tools, this data is compared with existing metabolites databases: Human Metabolome Database and METLIN. A database match shows a putative metabolite assignment. This result should be confirmed with retention time and MS/MS data of the samples. However, a database match does not show a fair number of metabolites that were detected by mass spectrometry (Patti, Yanes, &

Siuzdak, 2012). The last step of metabolomics analysis is data interpretation (Beger, 2013). The peaks that are shown in data are metabolite features and correspond to a detected ion with a unique mass-to-charge ratio and a retention time. Although metabolomic tools have been improved, there are still limitations. The masses of compounds that are detected in global analyses do not match the masses in metabolite databases. There are still many metabolites that are unknown (Patti, Yanes, & Siuzdak, 2012).

2.5.1 Cancer Metabolism

Cancer cells require genetic and epigenetic alterations to maintain tumor proliferation and metastasis (Wolf, Agnihotri, & Guha, 2010). Alteration of glycolytic metabolism is the most common biological process alteration in cancer (Agnihotri et al., 2013). Cancer cells synthesize carbohydrates, fatty acids, amino acids, and nucleotides for rapid proliferation (Beger, 2013) by aerobic glycolysis rather than mitochondrial oxidative phosphorylation (OXPHOS) and tricarboxylic acid (TCA) cycle, which is called the Warburg Effect. Figure 2.3 describes metabolic differences between normal and cancer cells. Normal cells produce pyruvate from glucose and go through the TCA cycle and the OXPHOS process with presence of oxygen to generate 36 ATPs per glucose. However, cancer cells convert most glucose to lactate despite the presence of oxygen and generate two ATPs per glucose. This anabolic process promotes rapid tumor proliferation (Marie & Shinjo, 2011). Glycolysis is a biochemical process that hydrolyzes glucose and produces two adenosine triphosphate (ATP), two NADH,

and two pyruvate (Berger et al., 2004). Tumors generate more than 50% of ATP by glycolysis even with the presence of oxygen. This is called aerobic glycolysis. As a result of aerobic glycolysis, an increased level of lactate that is produced from pyruvate in order to survive in microenvironment (Wolf et al., 2010). High level of lactate requires the activation of biological systems that equilibrate the intracellular pH level and may promote acidification of the tumor microenvironment (Santos & Schulze, 2012). In cancer environment, pyruvate dehydrogenase (PDH) activity is inhibited by pyruvate dehydrogenase kinase 1 (PDK1), a hypoxia-driven enzyme, and lactate dehydrogenase A (LHDA) activity is upregulated. Elaborated level of lactate causes acidic tumor environment and promotes tumor invasion (Marie & Shinjo, 2011).

GBM uses glycolysis and glutaminolysis to provide metabolic macromolecules for the cell proliferation. In GBM in vitro, 90% of glucose and 60% of glutamine produce lactate or alanine. GBM cells take advantage of this method to increase the cell division velocity by carbon incorporation into biomass. Glytaminolysis produces energy required for fatty acid synthesis by NADPH production (Marie & Shinjo, 2011). Grade three and four brain tumors often show alterations in phophoinositide 3-kinase (PI3K), EGF receptors (EGFR), vascular epithelial growth factor (VEGF), and PTEN signaling. According to the Cancer Genome Atlas Research Network, whole genome sequencing proved that abnormal signaling through the RTK/RAS/PI3K, p53, and retinoblastoma (RB) pathways play a critical role in glioblastoma development (Chinnaiyan et al.,

2012). Over-expressed metabolites in GBM include 2-hydroxyglutarate and isocitrate dyhydrogenase 1 mutation (Patti, Yanes, & Siuzdak, 2012).

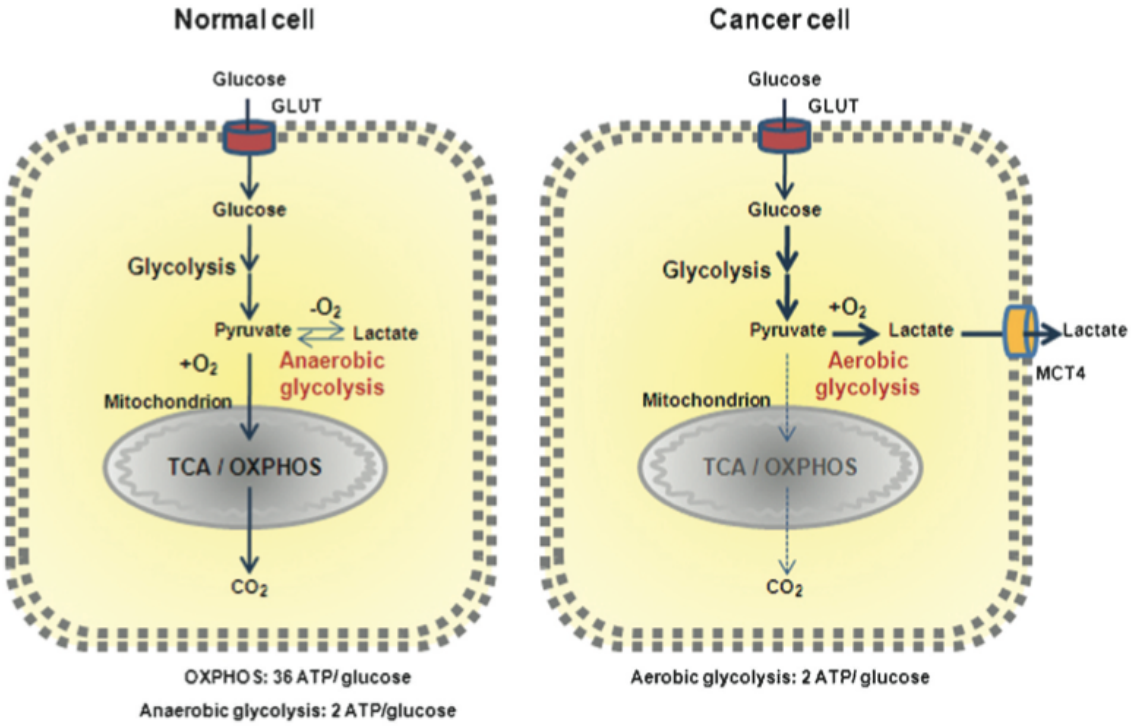


Figure 2.3. Metabolic differences between normal and cancer cells (Marie & Shinjo, 2011).

2.6 Lipidomics

Lipidomics is the systems-level analysis that identifies and quantifies pathways and networks of cellular lipids species. Lipidomics uses novel analytical technologies to understand the classes of lipids, changes in lipid metabolism and lipid-mediated signaling pathways, and interactions with other lipids, proteins, and other molecules (Wang et al., 2009; Tripathy, 2011). Lipids include fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides and phospholipids.

There are two main techniques that are utilized to analyze lipids species and interactions: liquid chromatography and mass spectrometry (MS) (Tripathy, 2011). This research study focuses on analysis of lipids using mass spectrometry, which is most frequently used in lipidomic research. Mass spectrometry in lipidomics provides higher quantitation performance and higher sensitivity than other exiting techniques. There are three main MS techniques, which are global lipidomic analysis, targeted lipidomic analysis, and novel lipid discovery. Global lipidomic analysis identifies and quantifies different types of lipids through a high throughput basis. This technique is used to analyze various pathways and lipid metabolism, trafficking, and homeostasis. Mapping techniques are used to investigate the spatial and temporal associations of lipids. Targeted lipidomics analysis is utilized to identify few specific lipid classes. Novel lipid discovery focuses on the discovery of novel lipid classes and molecular species (Tripathy, 2011)

2.6.1 Cancer Lipid Metabolism

Most lipids exist in cell membranes comprising the lipid bilayer. Due to the membrane organizing properties of lipids, lipids play many important roles in a cell, tissue and organ physiology. The main biological functions of lipids are as energy storage, structural components of cellular membranes, changes in cell membrane composition, cell signaling, endocrine actions, membrane trafficking, regulating membrane proteins, systemic lipid metabolism, lipid oxidation, and

biochemical reactions in the cells (Tripathy, 2011). Lipids are also co-sorted with proteins during the formation of transport carriers (Shevchenko & Simons, 2010). Brain tissue contains the highest amount of lipids compared to other organs. Human brain consists of 5-15% lipids, 70-83% water, and 7.5-8.5% proteins. The most abundant lipids are classified as cholesterol, phospholipids, and sphingolipids. White matter contains 27% cholesterol and 45% phospholipids, and gray matter contains 20% cholesterol and 67% phospholipids. When glioblastoma tumors are formed in the brain, lipid content decreases. (Campanella, 1992).

Lipid metabolism in cancer environment is regulated by oncogenic signaling pathways, and plays an important role in cancer initiation and progression (Zhang & Du, 2012). Lipid metabolism alteration may impact membrane structure, synthesis and degradation of lipids, homeostasis, and signaling functions. Lipid studies in cancer provide evidence that altered lipid metabolism supports tumor proliferation, differentiation, and motility (Santos & Schulze, 2012). Malignant transformation changes biosynthetic and bioenergetic tumor environments (Zhang & Du, 2012). Currently, most cancer lipid metabolism research studies focus on the increased level of fatty acid synthesis in the tumor environment. Figure 2.4 describes function of fatty acids stimulated by oncogenic signaling (Santos & Schulze, 2012). Enzymes that are involved in fatty acid synthesis, such as ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), are often over-expressed in many cancer types. Free

fatty acid levels are higher in more aggressive cancer cell lines and high-grade primary tumors.

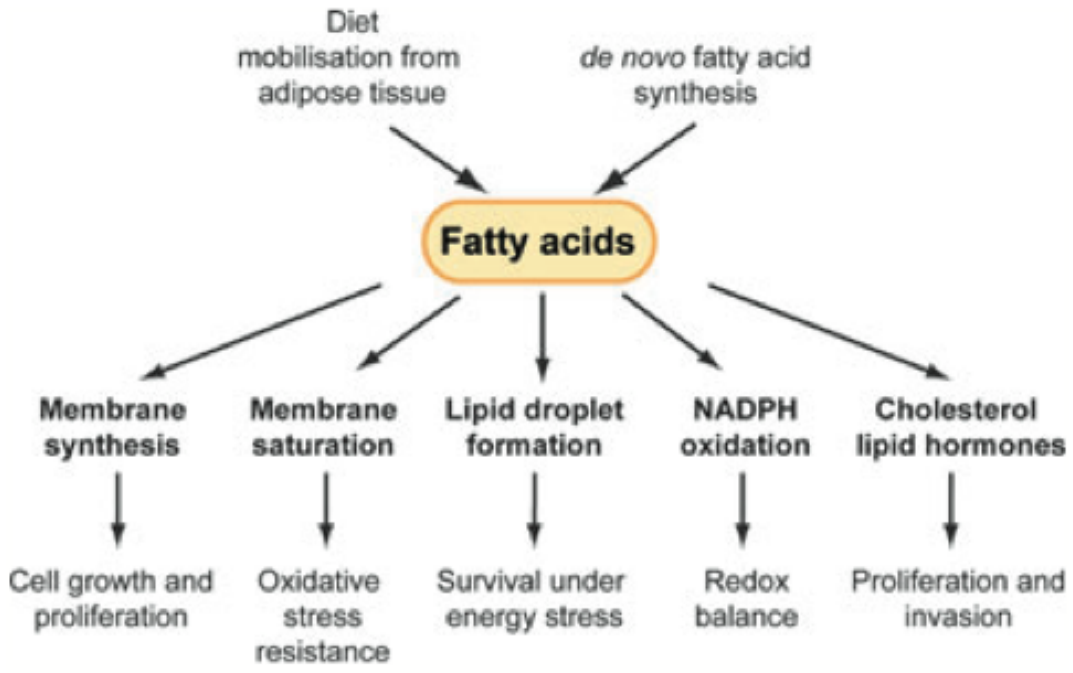


Figure 2.4. Functions of fatty acids in cancer cells (Santos & Schulze, 2012).

Alternation of lipid metabolism affects the phenotype of cancer cells. Large amount of lipids are required for forming the cell membrane when cancer cells proliferate (Santos & Schulze, 2012). Cancer cells rely on de novo endogenous lipid synthesis rather than exogenous dietary lipid synthesis. De novo lipogenesis has many important roles in tumor formation, such as membrane formation, lipid molecule signaling, protein modifications, and energy supply for rapid tumor proliferation. Fatty acids that are endogenously produced are often esterified to phospholipids to meet the needs of tumor formation, including structural building blocks, formation of a detergent-resistant membrane microdomain for tumor

signal transduction, polarization, intracellular trafficking, and migration of the cancer cells. Lipid molecules that mediate signal transduction, such as phosphatidic acid (PA), diacyl-glycerol (DAG), and lysophosphatidic acid (LPA), can activate signaling proteins or bind to G protein-coupled receptors (GPCRs) in order to control tumor proliferation, survival, and migration. Fatty acid can also go through β-oxidation to provide energy for cancer cells. In Akt-overexpressing glioblastoma, fatty acid oxidation is sufficient to support tumor cells and prevent glucose withdrawal-induced death (Zhang & Du, 2012).

Cancer cells frequently exist in hypoxic regions. GBM tumors are heterogeneous with pseudopalisading perinecrotic cells in modedate hypoxic region (pO_2 = 2.5-5%) and infiltrating cancer cells in normal brain oxygen conditions (pO_2 = 10%) (Wolf et al., 2010). When the oxygen level is low, hypoxia-inducible factors (HIFs) are activated. With alternations in Von Hippel-Lindau (VHL) tumor suppressor, HIF1-α and HIF2-α are stabilized even in presence of oxygen. Activation of HIFs may also occur by oncogenic pathways and deletion of p53. HIFs play many important roles in tumor growth process. Activated HIF upregulates vascular endothelial growth factor and encourages angiogenesis. It also promotes tumor cells to stabilize in hypoxic environment by anaerobic energy production. HIF1 upregulates the expression of FASN. HIF1 induces the hypoxia-inducible protein 2, a protein playing a role in deposition of neutral lipids into lipid droplets, in order to accelerate the accumulation of lipids. It also induces the peroxisome proliferator-activated receptor γ to support free fatty acid uptake and triacylglycerol production in liver and adipose tissue. In hypoxic

environments, glutamine plays an important role in lipid synthesis by providing carbon without mitochondrial activity (Santos & Schulze, 2012).

Lipid profiles are powerful information for drug and biomarker development. Lipid metabolism is potentially used as evidence of the identification of pathways. Analysis of lipids with other omics studies will provide better understanding of diseases that involve disruption of lipid metabolic enzymes and pathways (Tripathy, 2011).

2.6.2 Limitations

Lipidomic analysis has been difficult due to the specificity and complexity of lipid composition and the lack of techniques for the analysis (Tripathy, 2011). Regulation and the mechanisms of lipid compositional complexity associated with cell homeostasis are still poorly understood (Shevchenko & Simons, 2010). Moreover, mass spectrometry has limitations in the structural identification of lipids. Due to various classes and molecular species of lipids, it is extremely difficult to accommodate all lipid classes using existing current detection methods. In contrast to genomics and proteomics, current lipidomics technologies are not capable of predicting the number of individual lipid molecules in an organism. Therefore, mapping lipidomes is still unattainable using existing technologies (Tripathy, 2011). As a result of the lack of lipidome information, genomic and proteomic profiles have not been matched with knowledge of lipids. Despite the fact that identification of protein-lipid interactions is increasing, the structural mechanisms of a cell remain unclear (Shevchenko & Simons, 2010).

2.7 Summary

This chapter has provided an overview to the review of relevant literature. It provided information of glioblastoma, existing treatment options, advantages and disadvantages of the animal models, proteomics, metabolomics, and lipidomics in GBM studies. This chapter confirms that cancer model development and discovery of novel targeted therapy are desperately needed.

CHAPTER 3. METHODOLOGY

This study was conducted in order to identify significantly expressed lipids and correlate patterns of cancer metabolism that can be compared to glioblastoma metabolites and proteins. The workflow of lipidomics contained two main parts: biological sample preparation and data analysis (Figure 3.1). Biological sample preparation contains harvest and collection of GBM xenograft tumor (Figure 3.1-1A-B), and lipid extraction from the tumors (Figure 3.1-1C). The lipid fraction was taken and run through RHPLC (Figure 3.1-1D) front-coupled to Bruker 7T Mass Spectrometer in ESI-FTMS mode (Figure 3.1-1E). Data analysis contains raw MS data conversion into a compatible format (Figure 3.1-2A), data processing performed in mzMINE (Figure 3.1-2B), statistical analysis (Figure 3.1-2C), LIPID MAPS database search of top 30 most significant lipids (Figure 3.1-2D), and hierarchical clustering of the data (Figure 3.1-2E).

This chapter outlines the details of human GBM cell line information, xenograft models, biological sample extraction, the instrument, and data analysis tools.

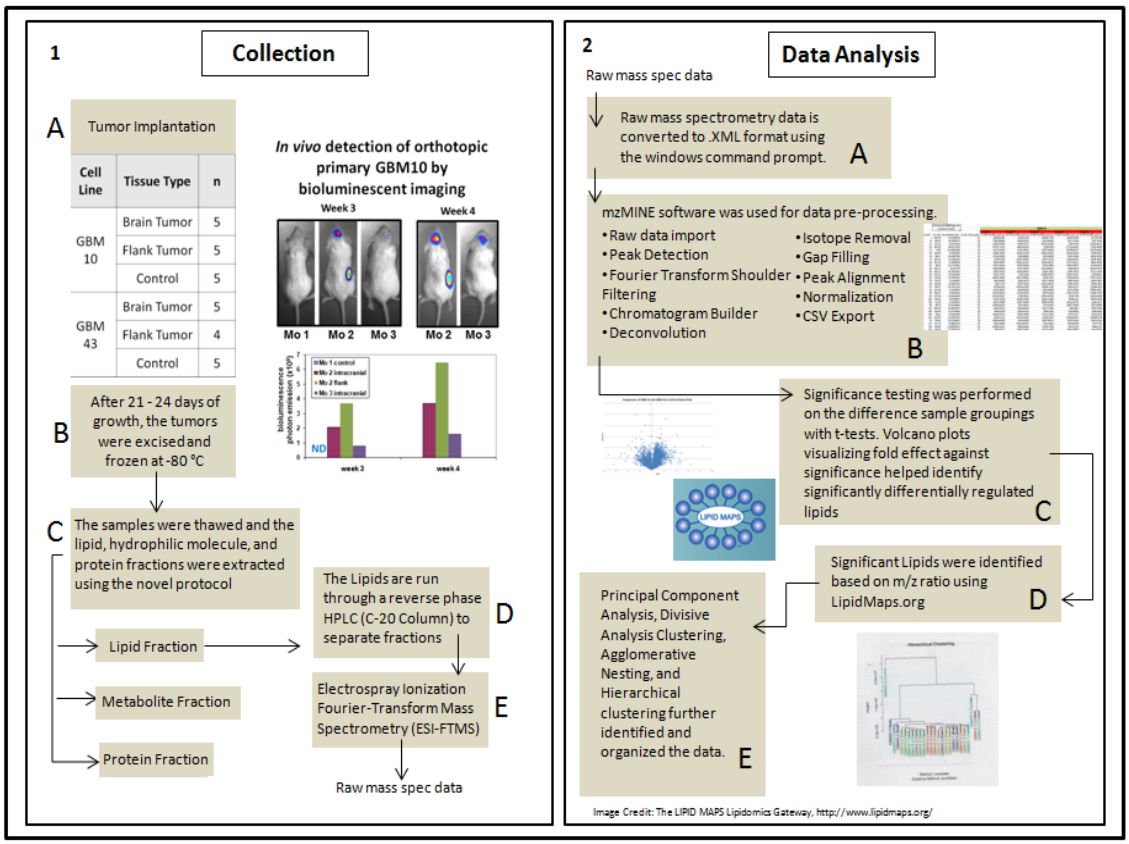


Figure 3.1. Overview of lipidomic analysis of GBM workflow.

3.1 Cell Lines and Cell Culture

Primary human GBM cell line GBM10 and GBM43 were surgically removed and provided from the Mayo Clinic, Rochester, Minnesota. Both cell lines were known to be resistant to temozolomide and several molecular properties were tested prior to this experiment. GBM10 and GBM43 both expressed normal EGFR and PTEN. GBM10 also has wild-type p53, tumor suppressor gene that is involved in many biological functions, yet GBM43 has mutant p53 (Carlson et al., 2011). Two types of in vivo mouse xenograft models (orthotopic and subcutaneous) of glioblastoma were needed and maintained at

the Indiana University School of Medicine. GBM10 and GBM43, which are resistant to current therapeutic options and actively investigated by the Brain Tumor Working Group of IUSM clinicians and science investigators from Purdue University, were propagated in the cerebrum of NOD/SCID mice. These cell lines were expanded in in vitro environment for approximately two to three weeks before being injected into the mouse xenograft models. $3x10^6$ cells were injected into right cerebral hemisphere and flank site of mice. Five mouse models were utilized per GBM cell type. Figure 3.2 summarizes the biological sample preparation process from in vitro cell expansion to protein, metabolite, and lipid extraction.

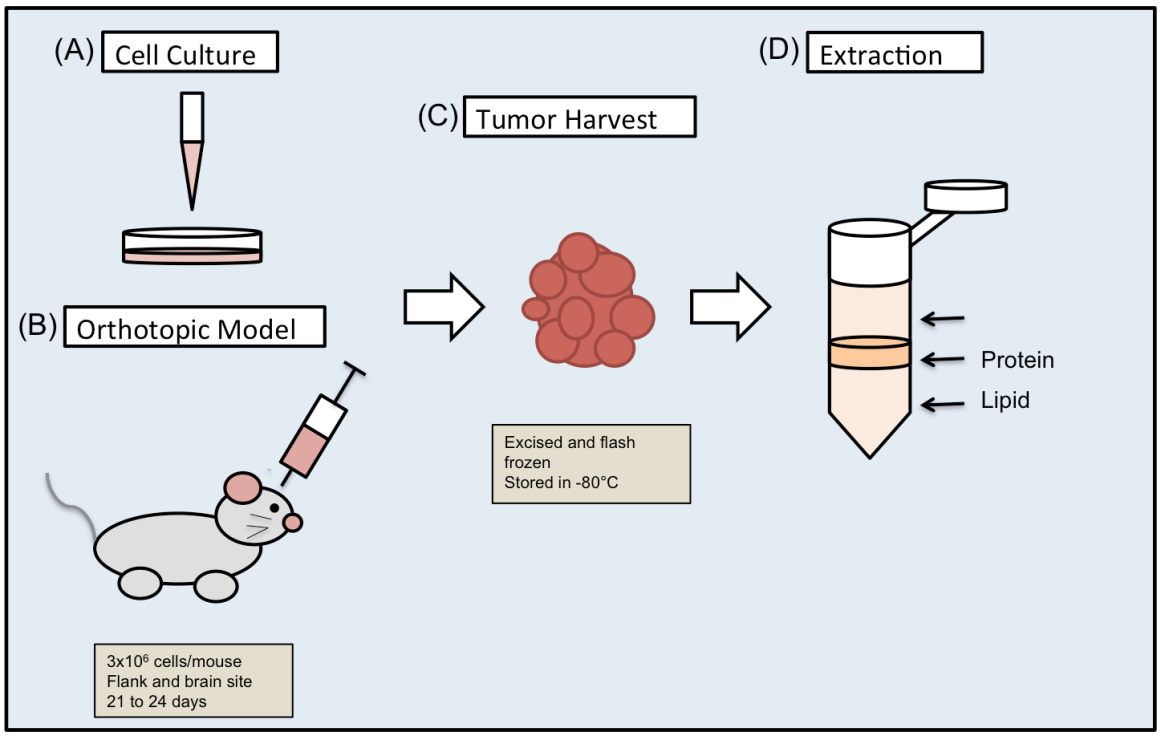
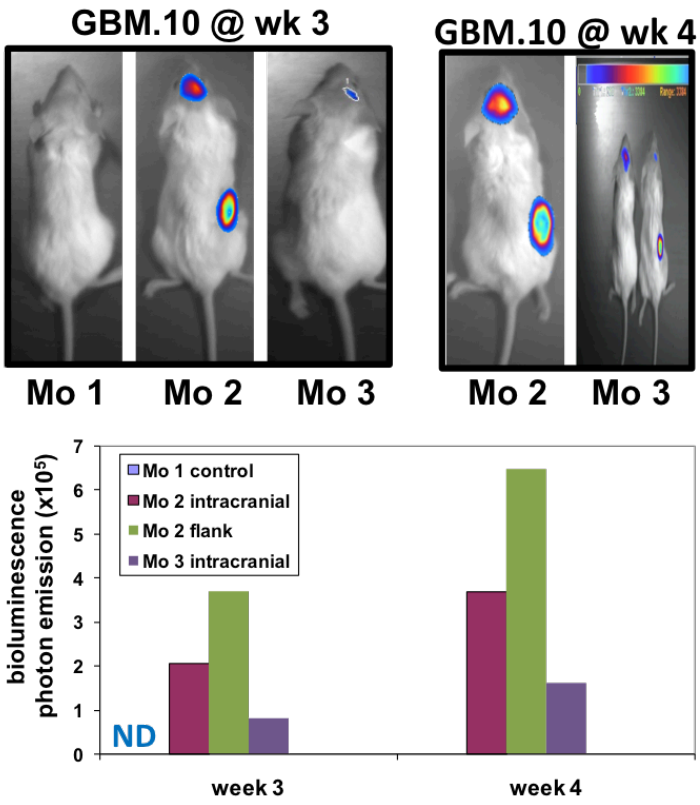


Figure 3.2. Overview of biological sample preparation process for mass spectrometry.

3.2 Mouse Orthotopic Models

NOD/Scid mice were obtained from the onsite breeding colony in the In Vivo Therapeutics Core at the Indiana University School of Medicine. All studies were reviewed and approved by the Indiana University Animal Care and Use Committee. GBM10 and GBM43 cells were implanted in the right flank in matrigel at 3 x 10^6 cells per mouse. For intracranial implantation, a digitalized stereotaxic delivery system was utilized (David Kopf Instruments, Model 5000 microinjection unit, Tujunga, CA). REF-1,2 for stereotaxic delivery of tumor cells, mice were placed under general anesthesia (ip injection of 16 mg/kg xylazine and 150 mg/kg ketamine) and positioned in the stereotaxic device. A digitalized drill assembly was used to bore a hole 0.3–mm in depth and 0.8-mm diameter in the cranium at a position 0.5-mm anterior and 1.2-mm lateral to the bregmal anatomical landmark. Tumor cells (2 x 10^5 in 10 ml of RPMI medium) were introduced slowly using a 10 ml Hamilton syringe at a depth of 3.5 mm at a rate of 2 µl/min. Once injection was complete, the needle was kept in place for at least 5 minutes and then slowly removed and the hole sealed with bone wax. The incision was closed with a wound clip. These models have been validated in past studies and the median survival is 21 to 24 days post-implantation of tumor cells. Figure 3.3 shows in vivo detection of orthotopic primary GBM10 tumors at week three and four by bioluminescent imaging. Mice with flank tumors were euthanized once tumors reached ~250 mm^3.



***In vivo* detection of orthotopic primary GBM. 10 by bioluminescent imaging.** GBM.10 cells that express the luciferase-EGFP fusion protein were implanted into the flank and brain, and the integrated photon flux determined over the tumor area via the NightOwl imaging system. GBM.10 xenografts (3 x 10^5 per site) were analyzed at weeks 3-4. Mo 1 served as a baseline control and was not implanted with tumor. ND = no bioluminescence detected in Mo1. Mo2 was implanted in the flank (2 x 10^6 cells) and the brain; Mo3 was implanted with cells in the brain only

Figure 3.3. In vivo detection of orthotopic primary GBM10 by bioluminescent imaging.

Tumors were excised, flash frozen, and stored at -80^{o}C. For mice with intracranial tumors, the mice were observed daily starting at 2 weeks and prior to reaching the pre-death endpoint were euthanized. Tumor tissue was excised from the right cerebrum and flash frozen and stored at -80^{o}C. Twenty-eight tissue samples of GBM10, GBM43 from brain and flank site, and control brains from total ten mouse models were listed in Table 3.1 & 3.2.

Table 3.1.

Quantity of tissue samples harvested from GBM xenograft models.

Cell line	Tissue type	Amount
GBM10	Brain tumor	5
	Flank tumor	5
	Control	5
GBM43	Brain tumor	5
	Flank tumor	4
	Control	5

Table 3.2.

Mouse xenograft tumor sample information.

Sample	Mouse ID	Cell Type	Tumor/Tissue Site
1	1	GBM10	Brain
2	1	GBM10	Flank
3	1	Control	Brain
4	2	GBM10	Brain
5	2	GBM10	Flank
6	2	Control	Brain
7	3	GBM10	Brain
8	3	GBM10	Flank
9	3	Control	Brain
10	4	GBM10	Brain
11	4	GBM10	Flank
12	4	Control	Brain
13	5	GBM10	Brain
14	5	GBM10	Flank
15	5	Control	Brain
16	M-1	Control	Brain

Table 3.2 (continued).

Mouse xenograft tumor sample information.

Sample	Mouse ID	Cell type	Tumor/Tissue site
17	M-1	GBM43	Brain
18	M-1	GBM43	Flank
19	M-2	Control	Brain
20	M-2	GBM43	Brain
21	M-2	GBM43	Flank
22	M-3	Control	Brain
23	M-3	GBM43	Brain
24	M-4	Control	Brain
25	M-4	GBM43	Brain
26	M-4	GBM43	Flank
27	M-5	Control	Brain
28	M-5	GBM43	Brain
29	M-5	GBM43	Flank

3.3 Protein, Metabolite and Lipid Extraction

Sample preparation of the mouse tumors prior to mass spectrometry was performed using a novel biomolecule extraction method to harvest the proteins, metabolites, and lipids in one simple and fast procedure.

10mg to 100mg of in vivo mouse GBM tumor tissues were placed in a low retention 2.0ml microcentrifuge tube and placed on ice. These tumor samples were mixed with 200µl of 75% MetOH in 0.15M NaCl. Approximately 50µl of grinding balls (ZrO; diameter ~0.5 mm) were added to each tube in order to homogenize the tumor tissues using a Next Advance Bullet Blender for two minutes. This step was repeated until the tissues were completely homogenized.

After the tissue homogenization, 20µl of suspension from each tube was transferred into the new 1.5ml microcentrifuge tubes. The homogenized samples were mixed with 180µl of 0.15M NaCl and 1ml of chloroform/methanol (2:1) with 0.01% BHT by vortex for two minutes, then incubated in room temperature for 30 minutes. After the incubation, the tubes were centrifuged for five minutes at 7,800xg. 250µl of the lower chloroform phase of the mixtures were transferred into the new tubes and labeled as 'lipid' fraction. These lipid fractions were stored in -80°C until mass spectrometry was performed. Remaining lipid phase of the samples were discarded and briefly vortexed. 100ul of suspension of each sample was transferred to the new microcentrifuge tubes. These suspensions in the new tubes were mixed with 100µl of MetOH, vortexed, and centrifuged for five minutes at maximum speed. The supernatant was transferred to the new tubes and labeled as 'Polar Metabolites' fraction. These polar metabolite fractions were also stored in -80°C. The pellets were washed with cold acetone by resuspending and centrifuging. After discarding acetone, the pellets were resuspended with the buffer. These mixtures were the 'protein' fraction.
For aniline labeling of polar metabolite fraction, the samples were dried under nitrogen. Then, dried samples were resuspended in 100µl of 0.15M NaCl.

3.4 Instrument

Mass spectrometry with front-end, reverse phase high-performance liquid chromatography (RHPLC) for molecule separation was performed on the sample fractions using electrospray ionization Fourier transform mass spectrometry (ESI-

FTMS; 7 T Bruker Instrument). The 26 lipid samples were randomly ordered and assigned corresponding designations. Thirty microliters of each sample were loaded into the appropriate vials and placed in the auto-sampling tray of an Agilent 1200 series HPLC running in the reverse phase with an ACE C8 silica column. The column utilized a solvent gradient as described in Table 3.3. The auto-sampler loaded 2 µl from each vial, running a methanol blank both prior to sampling and twice after each sample to ensure full elution of the sample from the column. After elution, each sample ran directly to the mass spectrometer. Electrospray ionization (ESI) reduces risk of fragmentation of the lipid during ionization. Meanwhile, FTMS provides high mass resolving power relative to other modes of mass spectrometry, allowing for enhanced detection of unique lipids. The mass spectrometer was run in both positive-ion mode and negative-ion mode to ensure a complete profile of lipids was achieved.

Table 3.3.

HPLC gradient parameters for lipid separation in reverse phase using Solvent A as 0.1 % formic Acid, 10 mM ammonium acetate in water and Solvent B as 0.1% Formic Acid, 10 mM ammonium acetate in acetonitrile: asoproponol in a 1:1 ratio.

Time (minutes)	% Solvent A	% Solvent B
0	70	30
1	70	30
25	0	100
45	0	100
47	70	30
60	70	30

3.5 Data Analysis

Profile data from the mass spectrometry was in the form of peak areas for each for recognized masses, which were converted to XML format and imported in the open source pre-processing software mzMine2. mzMine2 is a data processing tool for LC-MS data and designed for metabolomics profiles. This tool was utilized for peak detection, shoulder filtering, isotope removal, and gap filling (Pluskal et al., 2010) to compare control brain tissue to brain tumor tissue and flank tissue for the GBM10 and GBM43 samples independently. A series of homoscedastic t-tests comparing GBM10 and GBM43 brain, flank, and control tumors in all possible permutations identified the most significantly differentially regulated lipids. A selection of these were identified using LIPID MAPS, a web-based lipid database, which uses a text/ontology – based search to identify sub class, class, and common structure of a lipid within a variable m/z tolerance of 0.1-5 m/z.

3.6 Summary

This chapter has provided an overview to methodology of the research project, including the sample harvest, biomolecule extraction, maintenance and collection of data, and introduction of analysis tools.

CHAPTER 4. PRESENTATION OF THE DATA AND FINDINGS

The methodology of this project was performed to answer the research questions as introduced in Chapter 1. These questions were (1) what are the similarities and differences of molecular profiles between brain (orthotopic) and flank (subcutaneous) tumors in mouse xenograft model; and (2) are there biomarkers in GBM10 and GBM43 that are related to metabolic properties of GBM?

This chapter presents the mass spectrometry data that was processed with various bioinformatic and statistical tools, and the list of identified lipid classes. It also provides the comparison data between two different GBM cell lines and two different xenograft models. Detail interpretation of these results and further observations are described in Chapter 5.

4.1 Mass Spectrometry Data

Among all 26 samples, 11218 unique positive-ion mode peaks were quantified by mzMine2, representing an m/z ratio range of 244 to 1,800. To filter out insignificant peaks, a truncated data set was created by removing any peak detected in fewer than five samples, as there were five replicate samples in each

model grouping. This yielded 4,422 positive-ion mode peaks. A similar process for data ran in the negative-ion mode yielded 725 unique peaks.

Of the positive-ion mode data, 368 lipids were identified to be significantly different in lipid levels between GBM10 brain tumor tissue and the control tissue, 305 between GBM43 brain tumor tissue and the control tissue, and 1960 lipids between all brain tissue (both GBM10 and GBM43) and all flank tumor tissue. Of the negative-ion mode data, 149 lipids were significantly expressed between GBM10 brain tissue and the control tissue, 233 between GBM43 brain tissue and the control tissue, and 211 lipids between all brain tissue and all flank tissue. Summary of the number of significant lipids from different tissue types and the ratio of significantly decreased and increased lipids compare to control is shown in Table 4.1. More than 500 lipid species were significantly detected per different tissue type. More than 90% of these lipids were decreasingly expressed in both GBM10 and GBM43. This data clearly reported that GBM tumor tissue contains dramatically lower levels of lipid composition than normal brain tissue.

Table 4.1.

Differentially expressed significant lipids in different types of GBM tissues compare to control brain tissues.

		GBM10 Brain	GBM43 Brain	GBM10 Flank
Positive ion mode	Over-expressed	26	21	360
	Under-expressed	342 (92.9%)	284 (93.1%)	1566 (81.3%)
Negative ion mode	Over-expressed	2	0	4
	Under-expressed	147 (98.7%)	233 (100%)	153 (97.5%)

4.2 Significantly Expressed Lipids in GBM

Since there were over 500 identified lipids from different tissue types, the 30 most significantly identified lipids from each tissue type, which have p-value less than 0.05 and fold effect intensity greater than one, were plotted in order to visualize the most differentially expressed lipid classes, which are glycerophosphocholines, glycosphingolipids, glycerophosphoethanolamines, triradylglycerols, and glycerophosphoserines (Figure 4.1). The x-axis of the graph represents the structures of significantly identified lipids and the y-axis shows the fold effect change intensity of the lipids compare to control.

The positive ion profiles of glycosphingolipids showed similar lipid distribution between GBM10 and GBM43 brain tumors. Both types of brain tumors showed decreased level of glycosphingolipids. No flank tumor lipids were observed on the positive ion plot. However, the negative ion profiles did not show any lipids of GBM43, yet did show GBM10 and flank tumors (Figure 4.1 A). Mostly under-expressed flank tumor lipids were observed from the positive ion profiles. On the other hand, identified lipids from the negative ion profiles were equally distributed, mostly showing decreased level of lipids, among GBM10 brain tumors, GBM43 brain tumors, and all flank tumors. Most significantly identified lipids of the negative ion profiles among different tissue types were identified as glycerophosphocholines (Figure 4.1 B). Among the significant lipid classes, glycerohposphoserines were over-expressed throughout all different tissue types. GBM43 showed the highest fold effect change among all different tissue types, which is distinct since no fold effect of brain tumor lipids was

expressed higher than flank tumor. The negative ion data only showed the lipids from GBM43 and the flank tumors (Figure 4.1 C). The triradylglycerol plot showed equally distributed lipids from different tissue types. Some lipids of GBM10 and flank tumor were increased, yet majority levels of lipids were decreased (Figure 4.1 D). Apart from this distinction, casual observance implied that the flank tumor lipid profiles showed greater fold change intensity compare to the brain tumor profiles.

4.3 Orthotopic and Subcutaneous Xenograft Tumors

Significantly identified lipids between brain tumor tissues and flank tumor tissues were compared to investigate the similarities and differences. Table 4.2 represents the number of identified lipids in flank tumor compared to brain tumor tissues. There were a total of 1,960 significantly regulated positive ion lipids and 206 negative ion lipids in brain tumors compared to flank tumors. As the table shows, the majority of significant lipids were decreased in flank tumor tissues. Differentially expressed lipids between brain and flank tumors supports the pattern on Figure 4.1 and 4.2, which expresses m/z range and different fold effect change among the brain tumors and the flank tumors. Generally, lipidomic profiles of the flank tumors exhibit higher fold effect intensity than the brain tumors.

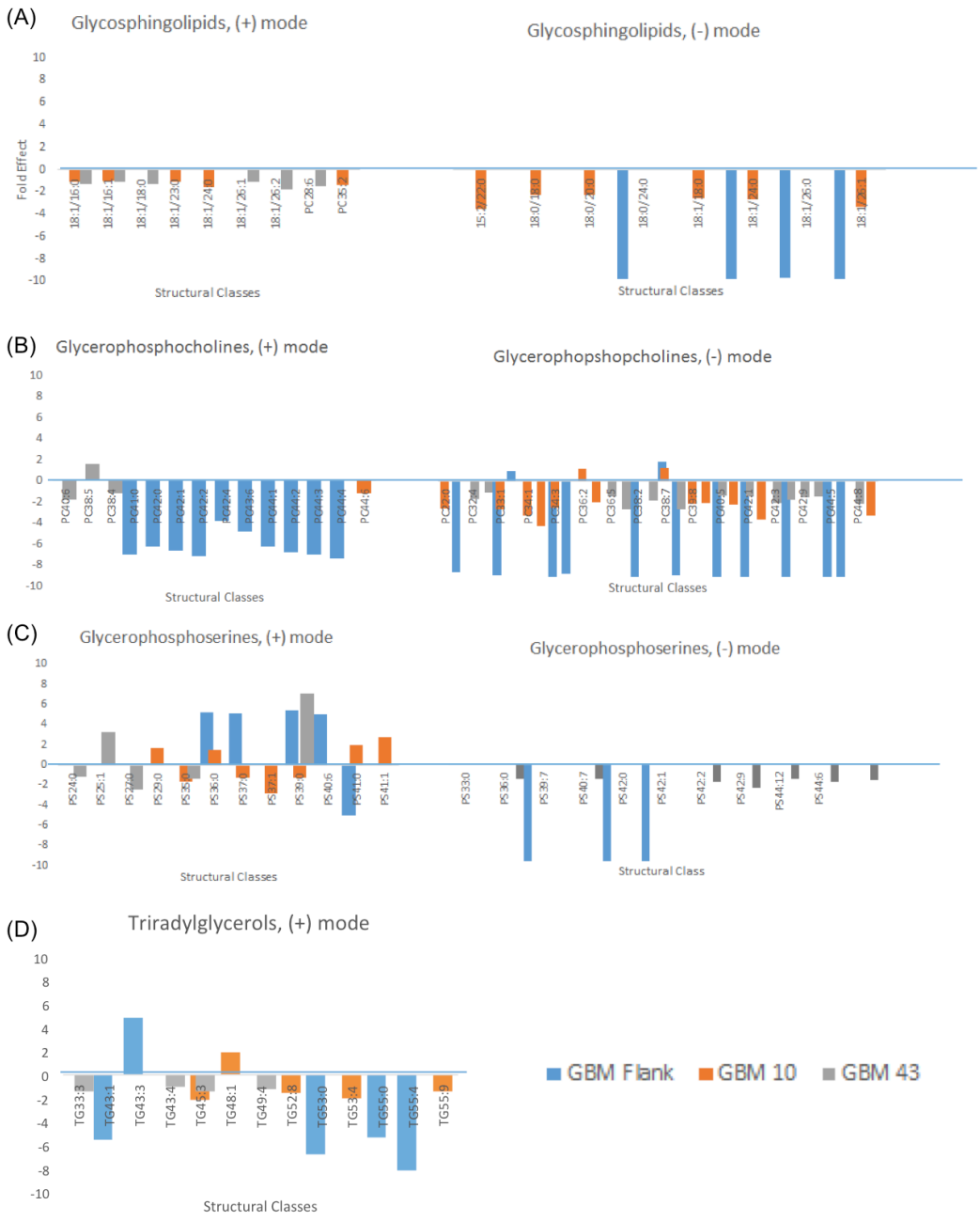


Figure 4.1. Top 30 significantly expressed lipid structures and lipid classes

Table 4.2.
Differentially expressed significant lipids between brain tumors and flank tumors.

	Number of lipids	
	Positive ion mode	Negative ion mode
Over-expressed	267	7
Under-expressed	1693	199

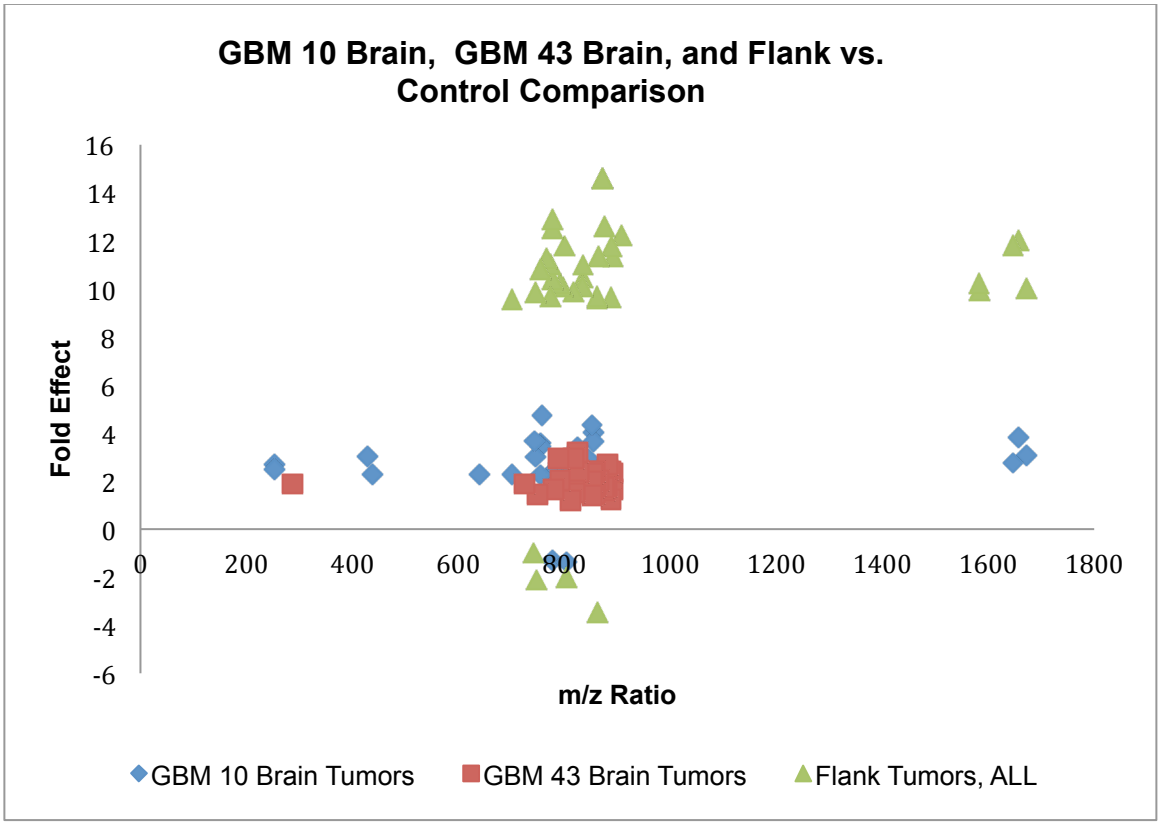


Figure 4.2. m/z and fold effect comparison of each tissue type.

4.4 Hierarchical Clustering of GBM Tumors

For quality assurance purposes, a series of statistical approaches were employed in order to identify any definite peculiarities with the results. In particular, a chief focus was to determine whether the data for the samples

demonstrated affinities consistent with the grouping of the samples. For this purpose, a set of clustering algorithms, Divisive ANAlysis (DIANA) and AGglomerative NESting Hierarchical Clustering (AGNES), were performed. To generate the clusters of the samples, m/z values and peak intensities from MzMine2 data were compared. As a result, the output from DIANA and AGNES showed similar patterns of clusters while these two algorithms have distinctly different paths to generate the output (Kaufman & Rousseeuw, 2009) and have a history of use with similar data (Gough et al., 2008).

As is evident from Figures 4.3 – 4.6, these disparate approaches produced closely related results. All the figures generated from DIANA and AGNES showed a propensity for the flank subjects to clearly cluster away from the other tissue types while the brain and control groups demonstrate a more closely clustered set of results. However, even in the brain and control groups, each group tended to segregate in the expected cohorts.

Based on the evidence rendered from using DIANA and AGNES, it is likely that the results achieve the aforementioned affinities, and accordingly the pattern mining efforts help to establish the viability of the experimental protocol.

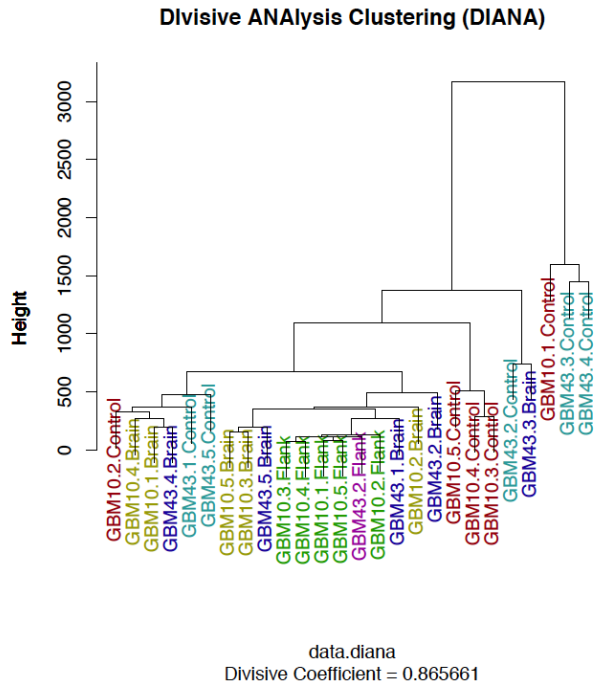


Figure 4.3. DIANA output from positive mode data.

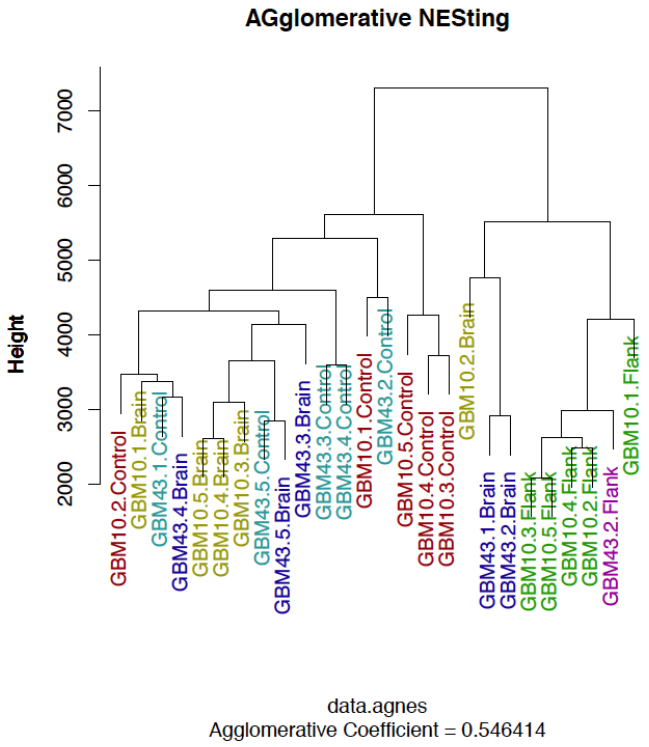


Figure 4.4. AGNES output from positive mode data.

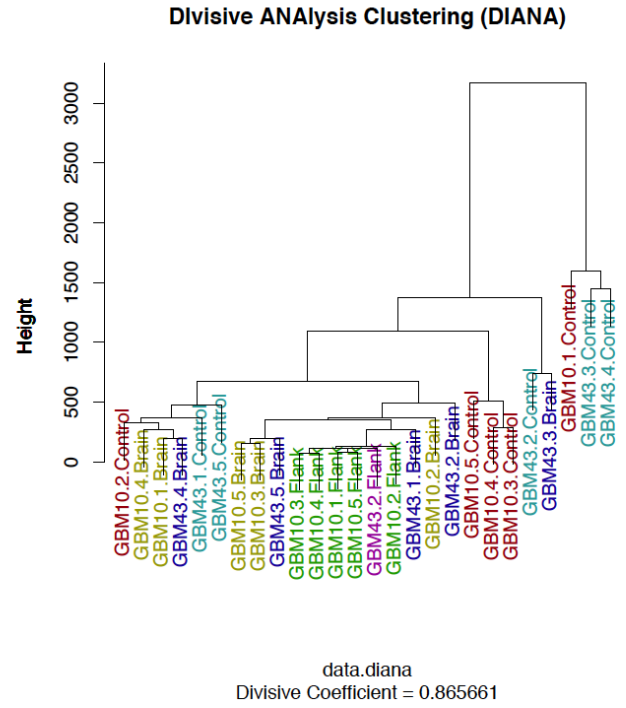


Figure 4.5. DIANA output from negative mode data.

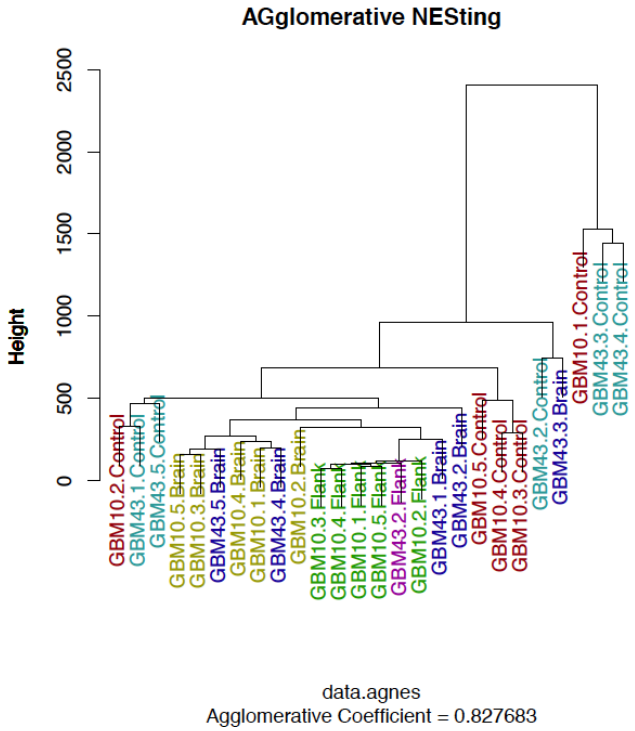


Figure 4.6. AGNES output from negative mode data.

4.5 Summary

This chapter has presented the data from mass spectrometry and various approaches to discover significance of the data. Employing bioinformatic tools and statistical tests identified significantly expressed lipids from different tissue types. Hierarchical clustering methods were also utilized to validate the data analysis approaches.

The next chapter draws upon the data presented in this chapter in order to define conclusions and discussion of the methods and results.

CHAPTER 5. DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

This chapter discusses the results generated by lipidomics workflow and mentions any observations that may affect the outcome of the experiment. It also summarizes the overall findings of this study and present potential future work to support the hypotheses that were drawn.

5.1 Discussion

5.1.1 GBM Cell Lines and Tumor Sizes

In vivo xenograft models were utilized to expand GBM tumor formation for the biomolecule extraction since cell viability and growth rate of GBM10 and GBM43, known to be resistant to temozolomide, were not consistently stable after approximately two to three weeks in in vitro environment. GBM cells were incubated in mouse xenograft models from 21 to 24 days in order to harvest appropriate size of tumors from viable mouse condition. Figure 5.1 represents the tumor sizes due to the different tissue types. The box plot of the tumor size showed that the size of GBM43 flank tumors was significantly smaller compared to other tumor tissues. GBM43 did not appeared on one of the mouse models so that only four GBM43 flank tumors were harvested instead of five. Moreover, three out of four GBM43 flank tumors were too small (1mg, 2mg and 2mg) for the

biomolecule extraction, which was designed for the tissue mass between 10mg and 100mg. Morphological differences between different GBM cell lines might be related to the tumor formation characteristics in xenograft models since GBM10 tends to form more localized tumors and GBM43 is known to have more invasive properties compare to GBM10.

Biomolecule extraction procedure also showed histological differences between GBM10 and GBM43. Flank tumor tissues were more difficult to homogenize compared to brain tumors and control samples. Control brain and brain tumor tissues were mostly homogenized on the first attempt. However, most of the flank tumors from GBM10 and GBM43 were homogenized up to five times in order to break down the tissue.

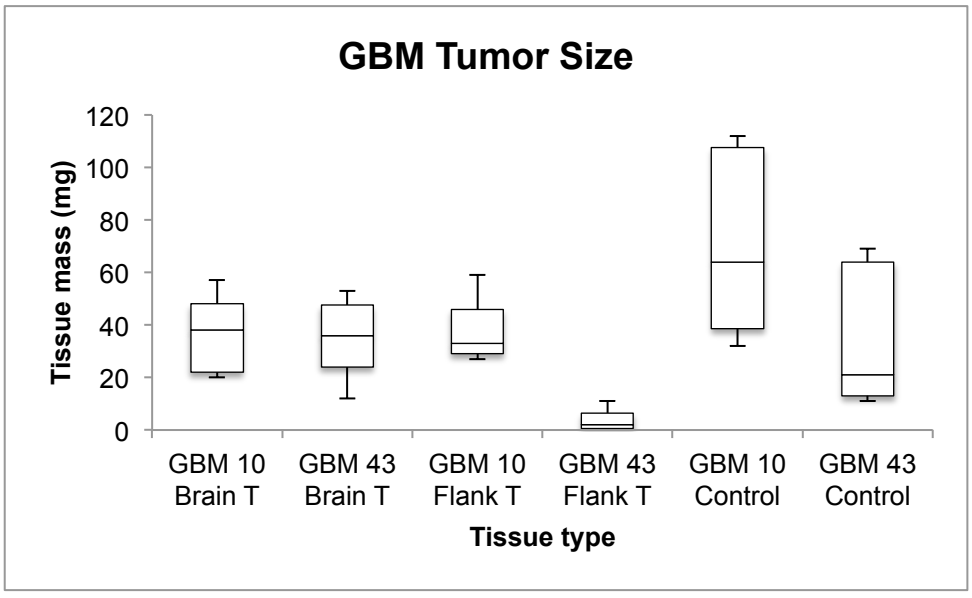


Figure 5.1. Tumor size comparison between different GBM tumors and control.

5.1.2 Biological Sample Preparation for Mass Spectrometry

The protocol for protein, metabolite, and lipid extraction was designed for the tissue size between 10mg and 100mg. This protocol was designed to overcome current limitations of sample preparation of metabolites and lipids. The extraction method was developed to extract proteins, metabolites, and lipids from one sample preparation. This method can be beneficial for the sample preparation in several ways. First, simple and minimal procedures can save sample preparation time, minimize degradation of metabolites, and lower sample loss during preparation period. Second, the samples from this protocol include a wide range of metabolites and proteins, which is beneficial for untargeted omic studies. Third, due to the simple and fast sample preparation steps, the sample preparation is easily reproducible. Reproducibility is important in omic studies, which use a large number of samples (Vuckovic, 2012).

5.1.3 Data Analysis

Quantitative analysis of the significantly differentially regulated lipids establishes patterns which indicate noteworthy differences between orthotopic and xenographic models of glioblastoma in mice. As previously mentioned, cursory analysis using t-tests and manual identification indicated that while general lipid profiles of flank tumors and brain tumors appeared to be in similar m/z range, comparison of lipid classes showed different lipid contents between different tissue types. Statistical validation method, hierarchical clustering, indicates distinct profiles of lipids in flank tumor tissues distinguished from both

brain tumor and brain control tissue, as brain tumor and brain control tissue cluster together. This evidence may suggest that brain tumors and control tissues were more similar to each other than flank tumor tissues. The clustering output may also imply that certain ‘background noise’ from surrounding tissue of the tumor (i.e. the variance in lipid composition of brain tissue and flank tissue) can account for a small portion of this clustering pattern.

Beyond the hierarchical clustering of the data, lipid identification introduces an element of uncertainty into the analysis. Because the mass spectrometer returned hundreds of significantly differentially regulated lipids, many of which shared nearly identical m/z ratios, power of manual analysis is influenced by both (1) the resolution of the LipidMaps database and (2) the size of the database (containing just over 37,500 unique lipids). The potential of fragmentation and creation of adducts within the ion generator, though roughly accounted for by mzMine2, adds further complication in manual identification of lipids. The instrument used, a Bruker 7-Tesla FT-MS, utilizing the *solariX* platform, provides mass accuracy on the magnitude of greater than under one part per million, which greatly increases the certainty of database returns on lipid identification. Likewise, the soft-ionization of the electrospray limits fragmentation and is compatible with front-coupled RHPLC used to separate lipids prior to analysis in the spectrometer.

5.2 Biological Implications

5.2.1 Lipid Studies and Decreased Level of Lipids in GBM

Our data showed that more than 90% of significantly identified lipids from in vivo brain and flank GBM tumors were under-expressed compared to the control brain tissue. On the other hand, the majority of the studies in cancer lipid metabolism have a tendency to focus on increased level of fatty acid synthesis (Zhang & Du, 2012). Lipidomic analysis has been difficult due to the specificity and complexity of lipid composition and the lack of techniques for the analysis (Tripathy, 2011). Regulation and the mechanisms of lipid compositional complexity associated with cell homeostasis are still poorly understood (Shevchenko & Simons, 2010). Moreover, mass spectrometry has limitations of the structural identification of lipids. Due to various classes and molecular species of lipids, it is extremely difficult to accommodate all lipid classes using existing current detection methods (Tripathy, 2011). Due to these difficulties in lipid studies, many research projects investigated the protein level of tumors to study lipid metabolism and not actual composition of lipids in cancer cells.

However, few number of lipid studies in gliomas examined the correlation between membrane lipid composition and malignancy of astrocytomas. A study that was published in 1992 measured membrane lipid changes among different grades of human gliomas using HPTLC. This study concluded that higher level of malignant glioma has significantly lower level of total plasma membrane lipids in tumor tissues (Campanella, 1992). More recent study using mass spectrometry to classify types of gliomas also showed that the profile of grade IV astrocytoma

total lipid abundance were lower than the low-grade astrocytomas (Eberlin et al., 2012).

Aggressive and high-grade primary cancer cells tend to have higher level of free fatty acid/ lipid droplets compared to normal or lower grade tumors. Monoacylgylcerol lipase (MAGL), a lipolytic enzyme that breaks down monoacylglycerols (MAGs) to produce glycerol and a free fatty acid, are also highly up regulated in aggressive form of cancer cells. Inhibition of MAGL showed that migration, invasion, and survival characteristics of cancer cells were inhibited in vitro and in vivo models (Zhang & Du, 2012). However, the mechanism of lipid droplets in cancer cell proliferation and survival is not well understood (Santos & Schulze, 2012). Our preliminary data of glioblastoma stem cell showed that these stem cells did not show the metabolic patterns of the Warburg Effect. If the cancer cell does not depend on glucose consumption, an alternative bioenergy mechanism should be utilized to maintain tumor cell survival (Liu, 2006).

5.2.2 Lipid Function and Fatty Acid Oxidation

Lipid functions in highly proliferating cancer cells are critical for building membranes for the cells and high level of lipids are required. The Warburg Effect explained that cancer cells utilize aerobic glycolysis rather than mitochondrial oxidative phosphorylation (OXPHOS) and tricarboxylic acid (TCA) cycle to maintain cancer environment (Tennant, Duran, & Gottlieb, 2010). According to our glucose flux biosensor experiment of glioblastoma stem cells, GBM stem

cells with rapid growth rate did not show significant amount of glucose utilization, which contradicts the Warburg Effect. This result supports the hypothesis that GBM may have a different cancer metabolism pattern other than the Warburg Effect. There are some cancer types that do not utilize high levels of glucose as cancer energy source. Prostate cancer does not depend on glucose for survival. Instead, prostate cancer shows increased uptake of fatty acids and upregulated beta-oxidation enzymes, which implies that prostate cancer cells depend heavily on fatty acid oxidation to maintain proliferation of cancer (Liu, 2006). Fatty acid oxidation also has a critical role in the proliferation and survival of leukemia (Samudio, et al., 2010). Energy stress and high glucose uptake can lead to increased fatty acid oxidation activity. In GBM, fatty acid oxidation contributes to energy production and resistance to oxidative and nutrient stress (Santos and Schulze, 2012).

There were few studies that examined the role of fatty acid oxidation in GBM to investigate the significance of fatty acid and cancer survival. These studies confirmed that stimulation of fatty aid oxidation provides sufficient energy for GBM to maintain cell survival and protect the cells from glucose deprivation. Moreover, more aggressive cancer cells lines expressed higher level of free fatty acid (Buzzai et al., 2005). This evidence of aggressive GBM and fatty acid oxidation can potentially explain the characteristics of GBM metabolism.

5.2.3 Signaling Pathways and Lipid Metabolism

Our lipidomics data showed significantly lower level of lipids in GBM, which does not support the cancer metabolism and signaling pathways. Figure 5.2 shows the signaling pathways that are related to lipid metabolism in cancer environment with mutant p53.

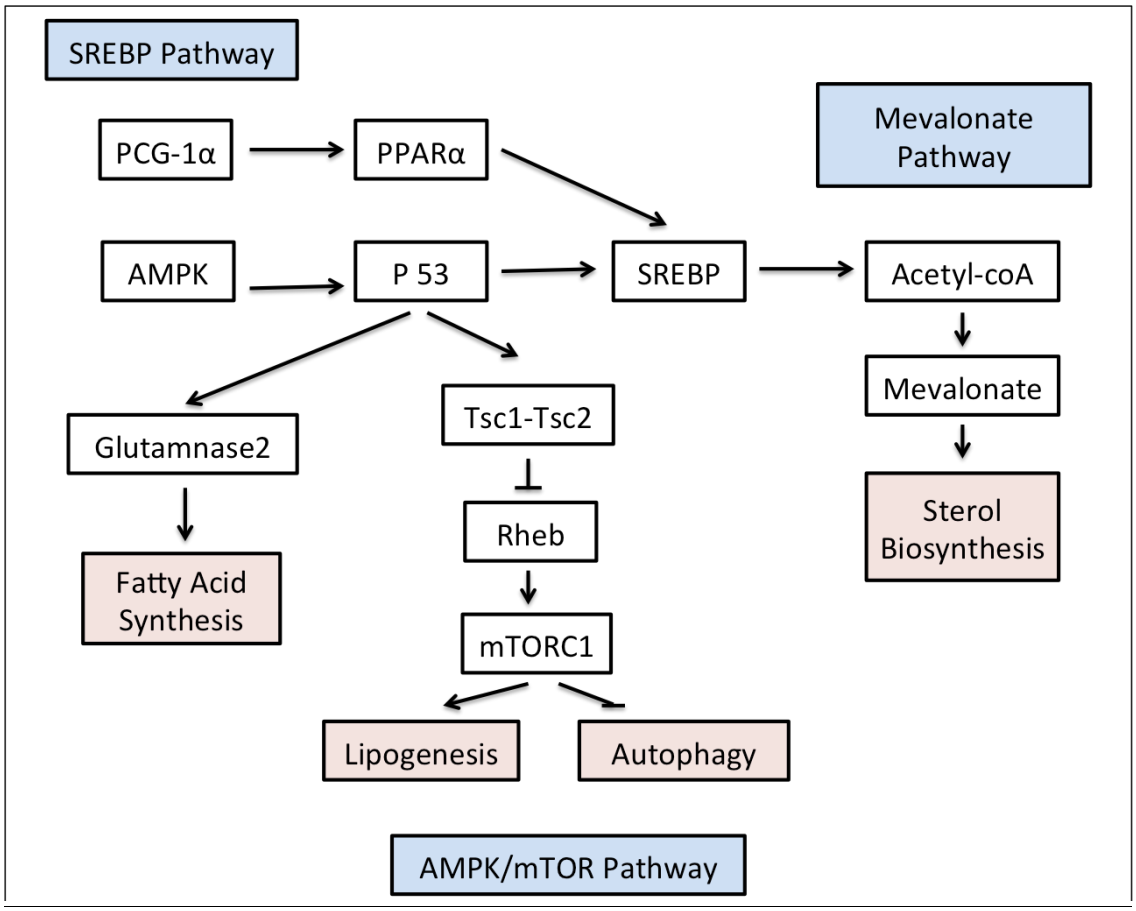


Figure 5.2. Signaling pathways and regulation of lipid metabolism in cancer

According to these cancer signaling pathways with p53 mutation, lipid synthesis level is upregulated in order to synthesis necessary lipids to maintain tumor growth. Tumor suppressor gene, p53, is the gene that is most commonly

alternated in many types of cancer and has various roles in metabolic regulations. Approximately 50% of cancer has p53 gene mutations (Maddocks & Vousden, 2011). Because of that reason, many cancer research projects are investigating mutation of p53 and alteration of metabolism in cancer. However, GBM shows distinct patterns of p53 mutation and tumor malignancy. Tumor suppressor gene, p53, mutations have been found in 25-30% of primary GBM and 60-70% in secondary GBM tumor. On the other hand, approximately 50% of lower grade glioma show p53 mutation, which explains that higher malignant forms of glioma have a less alternated p53 gene (Wang et al., 2009).

5.3 Conclusions

GBM10 and GBM43 cell lines were able to form appropriate size of brain tumor tissues with relatively healthy mice conditions. However, GBM43 tumors from subcutaneous sites were either too small or did not appear. Decreased cell growth and viability indicates that these cell lines may not be appropriate for long-term in vitro culture environment.

Overall, brain and flank tumors in mouse xenograft models showed decreased levels of lipids compare to control. Mass and charge ratio, m/z, ranges of identified lipids were also similar. However, there were profile differences in classes of significant lipids among these two xenograft tissues. Flank tumors also showed higher fold effect compared to brain tumors. The homogenizing procedure during the biomolecule extraction implied that histological characteristics of the brain tumors and flanks tumors were different. Hierarchical

clustering data analysis supported that control and brain tumor tissues were more closed related to each other than flank tumor tissues.

Figure 5.2 presents the classical lipid metabolism pathways in cancer environment with mutant p53, which does not match with the decreased lipid level from our data. Many studies suggested that aggressive tumors contain higher level of free fatty acids for energy source purpose. From this study, GBM cells had significantly lower level of lipid contents compared to the normal brain tissue. These results implied that GBM might use fatty acid oxidation as the main energy source in nutrient deprived cancer environments.

Our results do not follow the mutant p53 and lipid metabolism pattern, which is increased level of lipids. The lower percentage of p53 mutations in primary GBM compared to lower grade of gliomas and relationship between wild-type p53 and lipid metabolism suggest a hypothesis that GBM might manipulate biological functions of the wild-type p53 gene to metastasize and survive.

5.4 Future Recommendations

As mentioned in the Lipid Analysis section, we can compare the identified significant lipids with known pathways of tumor genesis. We anticipate these results will be supported by related metabolomic and proteomic data from the same set of samples, especially concerning potential down-regulation of lipid concentration in pathogenic tissue as a result of hypothesized oxidative lipid metabolism in glioblastoma tumor cells. We hypothesize proteomic data will return increased levels of enzymes implicated in both catabolism and anabolism

of lipids, while metabolic data will confirm decreased frequency of glycolytic pathways in favor of oxidative lipid metabolism.

LIST OF REFERENCES

LIST OF REFERENCES

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*, *422*(6928), 198-207.

Agnihotri, S., Burrell, K. E., Wolf, A., Jalali, S., Hawkins, C., Rutka, J. T., & Zadeh, G. (2013). Glioblastoma, a brief review of history, molecular genetics, animal models and novel therapeutic strategies. *Archivum immunologiae et therapiae experimentalis*, *61*(1), 25-41.

Alaiya, A. A., Franzen, B., Auer, G., & Linder, S. (2000). Cancer proteomics: from identification of novel markers to creation of artificial learning models fro tumor classification. *Electrophoresis, 21,* 1210-1217.

Argyle, D. J., & Khanna, C. (2006). *The biology of cancer:* small animal clinical oncology (pp. 31-53). Philadelphia: WB Saunders Company.

Barcus, C. (2010). *Protein-Protein Interactions: Stem Cells and Peptide Modified Silica Gels* (Master's thesis). Purdue University, West Lafayette, IN.

Beger, R. D. (2013). A review of applications of metabolomics in cancer. *Metabolites*, *3*(3), 552-574.

Berger, F., Gay, E., Pelletier, L., Tropel, P., & Wion, D. (2004). Development of gliomas: Potential role of asymmetrical cell division of neural stem cells. *The Lancet Oncology, 5*, 511-514.

Bonavia, R., Cavenee, W. K., & Furnari, F. B. (2011). Heterogeneity maintenance in glioblastoma: a social network. *Cancer research*, *71*(12), 4055-4060.

Brennan, C. W., Verhaak, R. G., McKenna, A., Campos, B., Noushmehr, H., Salama, S. R., Zheng, S., Chakravarty, D., Sanborn, J. Z., Berman, S. H., Beroukhim, R., Bernard, B., Wu, Chang-Jiun, Genovese, G., Shmulevich, I., Barnholtz-Sloan, J., Lihua, Z., Vegesna, R., Shukla, S. A., Ciriello, G., Yung, W. K., Zhang, W., Sougnez, C., Mikkelsen, T., Aldape, K., Bigner, D. D., Van Meir, E. G., Prados, M., Sloan, A., Black, K. L., Eschbacher, J., Finocchiaro, G., Friedman, W., Andrews, D. W., Guha, A., Iacocca, M., O'Neill, B. P., Foltz, G., Myers, J., Weisenberger, D. J., Penny, R., Kucherlapati, R., Perou, C. M., Hayes, D. N., Gibbs, R., Marra, M., Mills, G. B., Lander, E., Spellman, P., Wilson, R., Sander, C., Weinstein, J., Meyerson, M., Gabriel, S., Laird, P. W., Haussler, D., Getz, G. & Chin, L. (2013). The somatic genomic landscape of glioblastoma. *Cell*, *155*(2), 462-477.

Buzzai, M., Bauer, D. E., Jones, R. G., DeBerardinis, R. J., Hatzivassiliou, G., Elstrom, R. L., & Thompson, C. B. (2005). The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid β-oxidation. *Oncogene*, *24*(26), 4165-4173.

Campanella, R. (1992). Membrane lipids modifications in human gliomas of different degree of malignancy. *Journal of neurosurgical sciences*, *36*(1), 11.

Carlson, B. L., Pokorny, J. L., Schroeder, M. A., & Sarkaria, J. N. (2011). Establishment, maintenance, and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery. *Current Protocols in Pharmacology*, 14-16.

Chiang, A. C., & Massagué, J. (2008). Molecular basis of metastasis. *New England Journal of Medicine*, *359*(26), 2814-2823.

Chinnaiyan, P., Kensicki, E., Bloom, G., Prabhu, A., Sarcar, B., Kahali, S., Eschrich, S., Qu, X., Forsyth, P., & Gillies, R. (2012). The metabolomic signature of malignant glioma reflects accelerated anabolic metabolism. *Cancer research*, *72*(22), 5878-5888.

Choi, B. D., Archer, G. E., Mitchell, D. A., Heimberger, A. B., McLendon, R. E., Bigner, D. D., & Sampson, J. H. (2009). EGFRvIII-Targeted Vaccination Therapy of Malignant Glioma. *Brain Pathology*, *19*(4), 713-723.

Clarke, J., Butowski, N., & Chang, S. (2010). Recent advances in therapy for glioblastoma. *Arch Neurology, 67*(3), 279-283.

Cocola, C., Anastasi, P., Astigiano, S., Piscitelli, E., Pelucchi, P., VIlardo, L., Bertoli, G., Beccaglia, M., Veronesi, M. C., Sanzone, S., Barbieri, O., Reinbold, R. A., Luvoni, G. C., & Zucchi, I. (2009). Isolation of Canine Mammary Cells With Stem Cell Properties and Tumour-Initiating Potential. *Reproduction in Domestic Animals*, *44*, 214-217.

Collins, B. C., Lau, T. Y., O'Connor, D. P., & Hondermarck, H. (2009). Cancer proteomics—an evolving battlefield. *EMBO reports*, *10*(11), 1202-1205.

Eberlin, L. S., Norton, I., Dill, A. L., Golby, A. J., Ligon, K. L., Santagata, S., Cooks, R. G., & Agar, N. Y. (2012). Classifying human brain tumors by lipid imaging with mass spectrometry. *Cancer research*, *72*(3), 645-654.

Fountoulakis, M. (2001). Proteomics: current technologies and applications in neurological disorders and toxicology. *Amino Acids*, *21*(4), 363-381.

Galvão, E. R., Martins, L. M., Ibiapina, J. O., Andrade, H. M., & Monte, S. J. (2011). Breast cancer proteomics: a review for clinicians. *Journal of cancer research and clinical oncology*, *137*(6), 915-925.

Goldstein, I., & Rotter, V. (2012). Regulation of lipid metabolism by p53–fighting two villains with one sword. *Trends in Endocrinology & Metabolism*.

Gough, E., Oh, C., He, J., Riley, C., Buck, C., & Zhang, X. (2008). Proteome discovery pipeline for mass spectrometry-based proteomics. *BMC Bioinformatics*, *9*(Suppl 7), p21.

Grossman, S. A., Ye, X., Chamberlain, M., Mikkelsen, T., Batchelor, T., Desideri, S., Piantadosi, S. D., Fisher, J., & Fine, H. A. (2009). Talampanel with standard radiation and temozolomide in patients with newly diagnosed glioblastoma: a multicenter phase II trial. *Journal of Clinical Oncology*, *27*(25), 4155-4161.

Gupta, G. P., & Massagué, J. (2006). Cancer metastasis: building a framework. *Cell*, *127*(4), 679-695.

Hamahan, D., & Folkman, J. (1997). Patterns and emerging mechanisms of the angiogenesis switch during tumorgenesis. *Cell*, *86*(3), 354-364.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *cell*, *100*(1), 57-70.

Herbst, R. S., Heymach, J. V., & Lippman, S. M. (2008). Lung cancer. *The New England Journal of Medicine, 359*, 1367-1380.

Hicklin, D. J., & Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of Clinical Oncology*, *23*(5), 1011-1027.

Hondermarck, H. (2003). Breast Cancer when proteomics challenges biological complexity. *Molecular & Cellular Proteomics*, *2*(5), 281-291.

Jellinger, K. (1978). Glioblastoma multiforme: Morphology and biology. *Acta Neurochirurgica, 42*, 5-32.

Kaufman, L., & Rousseeuw, P. J. (2009). *Finding groups in data: an introduction to cluster analysis* (Vol. 344). John Wiley & Sons.

Kerr, J. F., Winterford, C. M., & Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, *73*(8), 2013-2026.

Khalil, A. A. (2006). Biomarker discovery: a proteomic approach for brain cancer profiling. *Cancer science*, *98*(2), 201-213.

Khasraw, M., & Lassman, A. B. (2010). Advances in the treatment of malignant gliomas. *Current oncology reports*, *12*(1), 26-33.

Kleihues, P., Burger, P. C., & Scheithauer, B. W. (1993). The new WHO classification of brain tumours. *Brain Pathology*, *3*(3), 255-268.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N. M., Pastorino, S., Purow, B. W., Christopher, N., Zhang, W., Park, J. K., & Fine, H. A. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer cell*, *9*(5), 391-403.

Lipsitz, D., Higgins, R. J., Kortz, G. D., Dickinson, P. J., Bollen, A. W., Naydan, D. K., & LeCouteur, R. A. (2003). Glioblastoma multiforme: Clinical findings, magnetic resonance imaging, and pathology in five dogs. *Veterinary Pathology, 40*, 659-669.

Liu, Y. (2006). Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. *Prostate cancer and prostatic diseases*, *9*(3), 230- 234.

Luptrawan, A., Liu, G., & Yu, J. S. (2008). Dendritic cell immunotherapy for malignant gliomas. *Reviews on Recent Clinical Trials*, *3*(1), 10-21.

Maddocks, O. D., & Vousden, K. H. (2011). Metabolic regulation by p53. *Journal of molecular medicine*, *89*(3), 237-245.

Marie, S. K. N., & Shinjo, S. M. O. (2011). Metabolism and brain cancer. *Clinics*, *66*, 33-43.

Martens, T., Laabs, Y., Günther, H. S., Kemming, D., Zhu, Z., Witte, L., Hagel, C., Westphal, M., & Lamszus, K. (2008). Inhibition of glioblastoma growth in a highly invasive nude mouse model can be achieved by targeting epidermal growth factor receptor but not vascular endothelial growth factor receptor-2. *Clinical Cancer Research*, *14*(17), 5447-5458.

Meredith Jr, J. E., Fazeli, B., & Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Molecular Biology of the Cell*, *4*(9), 953.

Mitchell, D. A., & Sampson, J. H. (2009). Toward effective immunotherapy for the treatment of malignant brain tumors. *Neurotherapeutics*, *6*(3), 527-538.

Miura, F. K., Alves, M. J. F., Rocha, M. C., Silva, R. D., Oba-Shinjo, S. M., & Marie, S. K. N. (2010). Xenograft transplantation of human malignant astrocytoma cells into immunodeficient rats: an experimental model of glioblastoma. *Clinics*, *65*(3), 305-309.

Murray, K. K., Boyd, R. K., Eberlin, M. N., Langley, G. J., Li, L., & Naito, Y. (2013). Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). *Pure & Applied Chemistry*, *85*(7).

Niclou, S. P., Fack, F., & Rajcevic, U. (2010). Glioma proteomics: status and perspectives. *Journal of proteomics*, *73*(10), 1823-1838.

Niedbala, R. S., Mauck, C., Harrison, P., & Doncel, G. F. (2009). Biomarker discovery: validation and decision-making in product development. *Sexually transmitted diseases*, *36*(3), S76-S80.

Oertel, J., von Buttlar, E., Schroeder, H. W., & Gaab, M. R. (2005). Prognosis of gliomas in the 1970s and today. *Neurosurgical focus*, *18*(4), e12-e12.

Ohgaki, H., Dessen, P., Jourde, B., Horstmann, S., Nishikawa, T., Di Patre, P. L., Burkhard, C., Schuler, D., Probst-Hensch, N. M., Maiorka, P. C., Baeza, N., Pisani, P., Yonekawa, Y., Yasargil, M. G., Lutolf, U. M., & Kleihues, P. (2004). Genetic Pathways to Glioblastoma A Population-Based Study. *Cancer research*, *64*(19), 6892-6899.

Pang, L. Y., & Argyle, D. J. (2009). Using naturally occurring tumours in dogs and cats to study telomerase and cancer stem cell biology. *Biochimica et Biophysica Acta, 1792*, 380-391.

Paoloni, M. C., & Khanna, C. (2007). Comparative oncology today. *Veterinary Clinics of North America: Small Animal Practice*, *37*(6), 1023-1032.

Paoloni, M., & Khanna, C. (2008). Translation of new cancer treatments from pet dogs to humans. *Nature Reviews Cancer*, *8*(2), 147-156.

Patti, G. J., Yanes, O., & Siuzdak, G. (2012). Innovation: Metabolomics: the apogee of the omics trilogy. *Nature reviews Molecular cell biology*, *13*(4), 263-269.

Pelloski, C. E., & Gilbert, M. R. (2007). Current treatment options in adult glioblastoma. *US Oncological Disease*, 105-109.

Pichlmeier, U., Bink, A., Schackert, G., & Stummer, W. (2008). Resection and survival in glioblastoma multiforme: an RTOG recursive partitioning analysis of ALA study patients. *Neuro-oncology*, *10*(6), 1025-1034.

Pike, L. S., Smift, A. L., Croteau, N. J., Ferrick, D. A., & Wu, M. (2011). Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1807*(6), 726-734.

Pluskal, T., Castillo, S., Villar-Briones, A., & Orešič, M. (2010). MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC bioinformatics*, *11*(1), 395.

Rai, A. J., & Chan, D. W. (2006). Cancer proteomics: serum diagnostics for tumor marker discovery. *Annals of the New York Academy of Sciences*, *1022*(1), 286-294.

Rana, M. W., Pinkerton, H., Thornton, H., & Nagy, D. (1977). Heterotransplantation of human glioblastoma multiforme and meningioma to nude mice. *Experimental Biology and Medicine*, *155*(1), 85-88.

Reardon, D. A., & Wen, P. Y. (2006). Therapeutic advances in the treatment of glioblastoma: Rationale and potential role of targeted agents. *The Oncologist, 11*, 152-164.

Richmond, A., & Su, Y. (2008). Mouse xenograft models vs GEM models for human cancer therapeutics. *Disease Models & Mechanisms*, *1*(2-3), 78-82.

Rodenhiser, D., & Mann, M. (2006). Epigenetics and human disease: translating basic biology into clinical applications. *Canadian Medical Association Journal*, *174*(3), 341-348.

Samudio, I., Harmancey, R., Fiegl, M., Kantarjian, H., Konopleva, M., Korchin, B., Kaluarachchi, K., Bornmann, W., Duvvuri, S., Taegtmeyer, H., & Andreeff, M. (2010). Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *The Journal of clinical investigation*, *120*(1), 142.

Santos, C. R., & Schulze, A. (2012). Lipid metabolism in cancer. *FEBS Journal*, *279*(15), 2610-2623.

Sausville, E. A., & Burger, A. M. (2006). Contributions of human tumor xenografts to anticancer drug development. *Cancer Research*, *66*(7), 3351-3354.

Shevchenko, A., & Simons, K. (2010). Lipidomics: coming to grips with lipid diversity. *Nature Reviews Molecular Cell Biology*, *11*(8), 593-598.

Simpson, R. J., Bernhard, O. K., Greening, D. W., & Moritz, R. L. (2008). Proteomics-driven cancer biomarker discovery: looking to the future. *Current opinion in chemical biology*, *12*(1), 72-77.

Simpson, R. J., & Dorow, D. S. (2001). Cancer proteomics: from signaling networks to tumor markers. *TRENDS in Biotechnology*, *19*, 40-48.

Speroni, L., de los Angeles Bustuoabad, V., Gasparri, J., Chiaramoni, N. S., Taira, M. C., Ruggiero, R. A., & del Valle Alonso, S. (2009). Alternative site of implantation affects tumor malignancy and metastatic potential in mice: Its comparison to the flank model. *Cancer biology & therapy*, *8*(4), 375-379.

Stupp, R., Goldbrunner, R., Neyns, B., Schlegel, U., Clement, P., Grabenbauer, G. G., Hegl, M. E., Nippgen, J., Picard, M., & Weller, M. (2007). Phase I/IIa trial of cilengitide (EMD121974) and temozolomide with concomitant radiotherapy, followed by temozolomide and cilengitide maintenance therapy in patients (pts) with newly diagnosed glioblastoma (GBM). *Journal Clinical Oncology*, *25*, 2000.

Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., Ludwin, S. K., Allgeier, A., Fisher, B., Belanger, K., Hau, P., Brandes, A. A., Gijtenbeek, J., Marosi, C., Vecht, C. J., Mokhtari, K., Wesseling, P., Villa, S., Eisenhauer, E., Gorlia, T., Weller, M., Lacombe, D., Cairncross J. G., & Mirimanoff, R. O. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The lancet oncology*, *10*(5), 459-466.

Stupp, R., Mason, W. P., Van Den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, G., Eisenhauer, E., & Mirimanoff, R. O. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New England Journal of Medicine*, *352*(10), 987-996.

Taillandier, L., Antunes, L., & Angioi-Duprez, K. S. (2003). Models for neuro-oncological preclinical studies: solid orthotopic and heterotopic grafts of human gliomas into nude mice. *Journal of neuroscience methods*, *125*(1), 147-157.

Tainsky, M. A. (2009). Genomic and proteomic biomarkers for cancer: a multitude of opportunities. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1796*(2), 176-193.

Tennant, D. A., Durán, R. V., & Gottlieb, E. (2010). Targeting metabolic transformation for cancer therapy. *Nature Reviews Cancer*, *10*(4), 267-277.

Tripathy, K. (2011). Lipidomics: A Promising area in. *Journal of Computer Science & Systems Biology*, *4*(5), 93-98.

Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., Alexe, G., Lawrence, M., O'Kelly, M., Tamayo, P., Weir, B. A., Gabriel, S., Winckler, W., Gupta, S., Jakkula, L., Feiler, H. S., Hodgson, G., James, C. D., Sarkaria, J. N., Brennan, C., Kahn, A., Spellman, P. T., Wilson, R. K., Speed, T. P., Gray, J. W., Meyerson, M., Getz, G., Perou, C. M., & Hayes, D. N. (2010). Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma Characterized by Abnormalities in PDGFRA, IDH1, EGFR, and NF1, *Cancer cell*, *17*(1), 98-110.

Vuckovic, D. (2012). Current trends and challenges in sample preparation for global metabolomics using liquid chromatography–mass spectrometry. *Analytical and bioanalytical chemistry*, *403,* 1523-1548.

Wang, C., Yang, J., & Nie, J. (2009). Plasma phospholipid metabolic profiling and biomarkers of rats following radiation exposure based on liquid chromatography–mass spectrometry technique. *Biomedical Chromatography*, *23*(10), 1079-1085.

Wang, Y., Yang, J., Zheng, H., Tomasek, G. J., Zhang, P., McKeever, P. E., Lee, E. & Zhu, Y. (2009). Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. *Cancer cell*, *15*(6), 514-526.

Wolf, A., Agnihotri, S., & Guha, A. (2010). Targeting metabolic remodeling in glioblastoma multiforme. *Oncotarget*, *1*(7), 567-577.

Yi, D., Hua, T. X., & Lin, H. Y. (2011). EGFR gene overexpression retained in an invasive xenograft model by solid orthotopic transplantation of human glioblastoma multiforme into nude mice. *Cancer investigation*, *29*(3), 229-239.

Zhang, Z., & Chan, D. W. (2005). Cancer proteomics: in pursuit of “true” biomarker discovery. *Cancer Epidemiology Biomarkers & Prevention*, *14*(10), 2283-2286.

Zhang, F., & Du, G. (2012). Dysregulated lipid metabolism in cancer. *World journal of biological chemistry*, *3*(8), 167.

Zhang, X. D., Qin, Z. H., & Wang, J. (2010). The role of p53 in cell metabolism. *Acta Pharmacologica Sinica*, *31*(9), 1208-1212.

PUBLICATION

PUBLICATION

Ha, S. J., Showalter, G., Rickus, J., Cai, S., Wang, H., Liu, W. M., Sarkaria, J. N., Springer, J., Adamec, J., Pollok, K. E., & Clase, K. L. (2013). Abstract B37: Proteomic and metabolomic analyses of glioblastoma using mass spectrometry. *Molecular Cancer Therapeutics*, *12*(11 Supplement), B37-B37.